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ACUTE AND CHRONIC TOXICITIES OF TNT AND RDX TO THE ENCHYTRAEID WORM, *ENCHYTRAEUS CRYPTICUS*, IN NATURAL SOILS

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We investigated individual toxicities of 2,4,6-trinitrotoluene (TNT) and hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) to the potworm *Enchytraeus crypticus* using the Enchytraeid Reproduction Test (ISO 16387:2004). Studies were designed to identify and characterize the predominant soil physicochemical parameters that affect the acute (mortality) and chronic (reproduction) toxicities of TNT or RDX to *E. crypticus*, and to generate ecotoxicological benchmarks for developing the ecological soil screening levels (Eco-SSLs) for risk assessments of contaminated soils. Soils with a wide range of physicochemical parameters included: Teller sandy loam (TSL), Sassafras sandy loam (SSL), Richfield clay loam (RCL), Kirkland clay loam (KCL), and Webster clay loam (WCL). Reproduction toxicity of TNT weathered-and-aged in soil, based on EC_{50} values, correlated strongly with soil organic matter and clay contents. The order of soil toxicity (from greatest to least) was: TSL > SSL > KCL = RCL > WCL. RDX was less toxic to *E. crypticus* compared with TNT in the same soil types. RDX toxicity for *E. crypticus* was greater in sandy loam soils compared with clay loam soils. Toxicity benchmarks established in TSL and SSL will be submitted to the U.S. Environmental Protection Agency Eco-SSL Workgroup for use in developing soil invertebrate-based Eco-SSLs for TNT and RDX.

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PREFACE

The work described in this report was authorized under Project No. SERDP CU-1210. The work was started in April 2002 and completed in August 2004.

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1. INTRODUCTION

Testing and training ranges are key elements in maintaining the capability, readiness, and interoperability of the Armed Forces. The substantially increased demand for training resources is usually associated with increased environmental impacts at training sites due, in part, to the release of energetic materials (EMs). Consequently, soil contamination with explosives, propellants, and related materials at many U.S. military installations is widespread, exceeding 15 million acres by some accounts (GAO, 2003). This contamination may pose risks to military personnel, the surrounding environment, and offsite human and ecological receptors, and thus may jeopardize the long-term sustainability of ranges and training sites. As of 2002, the U.S. Department of Defense (DOD) had identified 2307 sites with potential contamination by explosives (GAO, 2003). Army ammunition plants were identified as the most heavily contaminated sites (Kramer, 2002). By 2003, assessments had been completed for only 558 sites. The DOD has estimated that identifying, assessing, and cleaning up contamination from military munitions at such sites will cost from $8 billion to $35 billion and could take more than 75 years (GAO, 2003).

Among the most prevalent energetic residues found in soil at the impact areas of ranges were 2,4,6-trinitrotoluene (TNT) and hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX). Individually or in combination with one another and other chemical ingredients, TNT and RDX are used in a variety of explosive formulations, including Composition B (artillery, mortar), Composition C4 (demolition explosive), Tritonal and Composition H6 (Air Force bombs), Composition A4 (40 mm grenades), and Octol (antitank rockets) (Jenkins, 2007). Both explosives are solids at ambient temperatures (Monteil-Rivera et al., 2009) and are deposited on ranges as particles of solid material. TNT does not mineralize once exposed to the environment either aerobically or anaerobically, but it can be environmentally transformed to a variety of nitroaromatic species (Jenkins, 2007; Monteil-Rivera et al., 2009). RDX does not degrade aerobically to any extent and is persistent in surface soils (Jenkins, 2007; Monteil-Rivera et al., 2009). Consequently, concentrations of these explosives in soil have been reported to exceed 87,000 mg kg^{-1} for TNT (Simini et al., 1995) and 74,000 mg kg^{-1} for RDX (Best et al., 2006). Notwithstanding their persistence in soil, the effects of TNT and RDX on soil biota have not been sufficiently investigated (Kuperman et al., 2009a). As a result, scientifically defensible screening values, which could be used in ecological risk assessment (ERA), are not currently available for these explosives in soil (Kuperman et al., 2009b).

Assessment and protection of the terrestrial environment at defense installations can be advanced by developing and applying scientifically based ecological soil screening levels (Eco-SSLs; http://www.epa.gov/ecotox/ecossl/; last accessed October 2012) for energetic materials released into upland aerobic soil environments (USEPA, 2005). The Eco-SSLs are concentrations of contaminants in soil that are protective of ecological receptors that commonly come into contact with soil or ingest biota that live in or on such soils. These values can be used in the screening level ERA (SLERA) to identify those contaminants that are not of potential ecological concern in soils and thus do not require further evaluation in the baseline ecological

risk assessment (BERA), potentially resulting in cost savings during ecologically based site assessments and remedial investigations. Use of Eco-SSL values can help site managers to distinguish those sites that do not pose significant environmental risks from those that do, prioritize contaminated sites by the level of risk posed, quantify the relative risks at each site, and decide whether further investigation in a BERA is merited to determine appropriate remedial actions.

Eco-SSLs are derived using published data generated from laboratory toxicity tests with different test species that are relevant to soil ecosystems. Our extensive literature review (Kuperman et al., 2009a) showed that, despite considerable attention to assessing ecotoxicity of energetics, the available data for TNT and RDX were insufficient to generate Eco-SSL values for soil invertebrates. To fill the existing data gaps, we conducted definitive studies designed to specifically meet the U.S. Environmental Protection Agency criteria (USEPA, 2005) for derivation of toxicity benchmarks acceptable for Eco-SSL development and expand the ecotoxicological data set, which can aid site managers in the knowledge-based decision-making process of securing the sustainable use of testing and training installations.

In previous studies, we investigated the effects of TNT or RDX on the enchytraeid worm (potworm) *Enchytraeus crypticus* and established toxicity benchmarks for these explosives using a natural Sassafras sandy loam (SSL) soil (Kuperman et al., 2003, 2004a, 2005). This soil had sufficiently low concentrations of organic matter and clay to fulfill the U.S. EPA requirement for using soil with characteristics that support high relative bioavailability of organic pollutants for developing realistic conservative Eco-SSL values (USEPA, 2005). However, exposure to RDX in SSL soil did not affect survival of adult potworms even at concentrations as high as 21,400 mg kg^{-1}. Juvenile production was affected by RDX, but the toxicity was relatively low in SSL, with EC_{20} estimates of 3715 and 8800 mg kg^{-1} for RDX freshly amended (FA) and weathered-and-aged (W-A) in soil, respectively. (The EC_{20} value is the TNT or RDX concentration producing a 20% decrease in the measurement endpoint. The EC_{20} parameter based on the reproduction endpoint is the preferred parameter for deriving Eco-SSL values.) Although ecotoxicological data determined in those studies can be representative of potential exposure effects in soils with similar chemical bioavailability conditions, such data can over- or underestimate the toxicities of TNT or RDX in soil types with properties that contrast with those of SSL. Studies with multiple natural soil types representing a range of soil parameters were required to confirm the toxicity data established with SSL. This was accomplished by determining ecotoxicological benchmarks for TNT and RDX in a different natural soil (Teller sandy loam [TSL]) with properties that support conditions of high relative bioavailability (USEPA, 2005), similar to those of SSL, and conducting toxicity testing with additional natural soils to extend the range of soil physicochemical characteristics, hypothesized to affect EM toxicity (USEPA, 2005), to identify the relationships among predominant soil physicochemical parameters and the toxicity of TNT or RDX for *E. crypticus* survival and reproduction.

2. MATERIALS AND METHODS

2.1 Soil Collection and Characterization

Toxicity testing was conducted using soils with relatively wide ranges of physicochemical characteristics. These soils included:

- TSL, fine-loamy, mixed, active, thermic Udic Argiustoll collected from agricultural land of the Oklahoma State University Perkins Experiment Station, Payne County, OK;
- SSL, fine-loamy, siliceous, semiactive, mesic Typic Hapludult collected from an open grassland field in a coastal plain on the U.S. Army Aberdeen Proving Ground in Harford County, MD;
- Kirkland clay loam (KCL), fine, mixed, superactive, thermic Udertic Paleustoll collected from Payne County, OK;
- Richfield clay loam (RCL), fine, smectitic, mesic Aridic Argiustoll collected in Texas County, OK; and
- Webster clay loam (WCL), fine-loamy, mixed, superactive, mesic Typic Endoaquoll collected in Story Country, IA.

The qualitative relative bioavailability (QRB) scores for organic chemicals in natural soils were considered "very high" for TSL and SSL, "medium" for KCL and WCL, and "low" for RCL according to the Eco-SSL criteria (USEPA, 2005). During soil collection in the field, vegetation and the organic horizon were removed, and the top 12 cm of the A-horizon were then collected. Soil was sieved through a 5 mm mesh screen, air-dried for at least 72 h, mixed periodically to ensure uniform drying, passed through a 2 mm sieve, then stored at room temperature before use in testing. The respective soils were then analyzed for physical and chemical characteristics. Results of these analyses are presented in Table 1.

Table 1. Physical and Chemical Characteristics of Soils Used in Toxicity Testing

Soil Parameter	TSL	SSL	KCL	RCL	WCL
Sand, %	65	70	37	30	33
Silt, %	22	13	34	43	39
Clay, %	13	17	28	28	28
Texture	Sandy loam	Sandy loam	Clay loam	Clay loam	Clay loam
Cation exchange capacity (CEC), cmol kg^{-1}	4.3	5.5	10.3	27.6	20.8
Organic matter, %	1.4	1.2	2.6	3.3	5.3
pH	4.4	5.2	6.4	7.4	5.9
Water-holding capacity (WHC), %	13	18	20	21	23
QRB*	Very high	Very high	Medium	Low	Medium

*Based on QRB scores for nonionizing organic contaminants in natural soils (USEPA, 2005).

2.2 Chemicals and Reagents

EMs used in the studies included 2,4,6-TNT (Chemical Abstracts Service [CAS] no. 118-96-7; purity, 99.9%) and RDX (CAS no. 121-82-4; purity, 99%). These EMs were obtained from Defense Research and Development Canada-Valcartier (Quebec City, Quebec, Canada). Beryllium sulfate ($BeSO_4$ $4H_2O$; CAS no. 7787-56-6; purity, 99.99%) was used as the positive control in all toxicity tests. High-performance liquid chromatography (HPLC)-grade acetone (CAS no. 67-64-1) was used to prepare individual EM solutions prior to soil amendments. Acetonitrile (ACN; CAS no. 75-05-8; HPLC grade), methanol (CAS no. 67-56-1; chromatography grade; purity, 99.9%), and calcium chloride ($CaCl_2$; CAS no. 10043-52-4; reagent grade) were used for the soil extractions and in analytical determinations by HPLC. Certified standards of TNT and RDX (AccuStandard, Inc., New Haven, CT) were used in HPLC determinations. ASTM Type I water (18 MΩ cm at 25 °C; ASTM, 2004a) was used throughout the toxicity studies. It was obtained using Milli-RO 10 Plus followed by Milli-Q PF Plus systems (Millipore, Bedford, MA). The same grade of water was used throughout the analytical determinations. Glassware was washed with phosphate-free detergent and sequentially rinsed with tap water, ASTM Type II water (>5 MΩ cm at 25 °C), analytical reagent-grade nitric acid 1% (v/v), and ASTM Type I water.

2.3 Soil Amendment Procedures

Studies were performed separately and independently for TNT or RDX in FA soil and in W-A soil, to determine toxicity benchmark values for TNT or RDX in each exposure type. During the soil amendment procedure, TNT or RDX was amended into separate aliquots of soil using an organic solvent (acetone) as a carrier. This was necessary to distribute the TNT or RDX evenly and uniformly to a large soil surface area, which would have been difficult to achieve if solid chemical crystals had been added to soil. The exceptions were 10,000 and 20,000 mg kg^{-1} treatments, which substantially exceeded solubility levels of RDX in acetone carrier. These treatments were prepared by adding appropriate amounts of dry crystalline RDX to clean TSL or SSL soil and the same acetone volume as was used in preparation of other treatments. Carrier control soils were amended with acetone only. Soil was spread to a thickness of 2.5 cm. Individual EMs were dissolved in acetone in glass volumetric flasks then pipetted across the soil surface, ensuring that the volume of solution added at any one time did not exceed 15% (v w^{-1}) of the soil dry mass. After the solution was added, the volumetric flask was rinsed twice with a known volume of acetone, and the acetone rinsate was also pipetted onto the soil. If the total volume of solution needed to amend the soil exceeded 15% (v w^{-1}), the solution was added in successive stages. The acetone was allowed to evaporate between additions for a minimum of 2 h within a darkened chemical hood. The same total EM/acetone solution volume at different EM concentrations was added to every treatment, to equal the volume required to dissolve EM at the greatest dissolved concentration amended. To prevent photolysis of the EM, amended soil was air-dried overnight (minimum of 18 h) in a darkened chemical hood. Each soil treatment sample was then transferred into a fluorocarbon-coated high-density polyethylene container and mixed for 18 h on a three-dimensional rotary soil mixer. After the soil samples were mixed, those containing the FA EM were hydrated with ASTM Type I water to 100% of the respective WHC of each soil and allowed to moisture-equilibrate for 24 h. Enchytraeid worms were then added for commencement of toxicity testing. Additionally, prior to additional toxicity testing, samples

of each FA soil were hydrated with ASTM Type I water to 60% of the respective WHC to initiate weathering-and-aging.

2.4 Weathering-and-Aging Explosives in Soil

Explosives in soils at many contaminated sites have been subjected to weathering-and-aging processes onsite for many years. Therefore, special consideration was given to weathering-and-aging of EM in soil for assessing the toxicities of TNT or RDX to *E. crypticus*, to provide appropriate benchmark data for Eco-SSL development. Standardized methods for weathering-and-aging of explosives in soil are not available. We have developed procedures that simulate, at least partially, the weathering-and-aging processes for chemicals in soil. These procedures allowed us to more accurately approximate the exposure conditions for soil biota in the field, compared with tests conducted with FA chemicals or tests conducted following a short equilibration period (e.g., 24 h) (Kuperman et al., 2003, 2005, 2006a, 2006b, 2006c, 2006d; Simini et al., 2003, 2006). In open glass containers, air-dried soil batches were amended with several concentrations of either TNT or RDX and initially hydrated with ASTM Type I water to 60% of the WHC of each soil. Soil was then subjected to alternating hydrating and air-drying cycles at ambient temperatures in a greenhouse. All soil treatments were weighed and readjusted to their initial mass by adding ASTM Type I water each week. Any soil surface crusting that formed during the week was broken with a spatula before water was added. After completion of the EM weathering-and-aging procedures, all soil treatments were brought to 100% of the WHC of each soil 24 h before commencement of toxicity tests. The effects of weathering-and-aging of TNT or RDX in soil on toxicity to *E. crypticus* were investigated by comparing test results for EMs W-A in soils with results obtained using soils with FA EMs.

Soil treatments with TNT concentrations representing low, intermediate, and high levels were monitored periodically during the weathering-and-aging process to determine the times when TNT concentrations were effectively stabilized or had declined to ≤5% of the initial concentration in FA soil treatments with the highest rate of decrease. Nominal TNT concentrations selected for monitoring in these studies were: 20, 100, 200, and 300 mg kg in TSL; 50, 100, 200, and 400 mg kg^{-1} in SSL or KCL; 5, 25, 100, and 500 mg kg^{-1} in RCL; and 40, 100, 200, and 400 mg kg^{-1} in WCL. The respective times determined for each TNT–soil pairing were then designated for terminating the weathering-and-aging procedures within treatments for that respective soil, and commencement of the corresponding definitive toxicity tests.

Previous studies have shown that RDX does not significantly degrade under aerobic conditions, and that toxicity to soil invertebrates did not change significantly ($p \leq 0.05$) when RDX was subjected to the weathering-and-aging process in soil (Dodard et al., 2005; Kuperman et al., 2003, 2004a; Simini et al., 2003). Therefore, soil concentrations of RDX were not monitored during the 3 month weathering-and-aging process. RDX concentrations were analytically determined in each soil treatment immediately before toxicity testing was started.

2.5 ACN Extraction of TNT and RDX from Soil

Concentrations of TNT and RDX were analytically determined in all control and treated soils, in triplicate, at the beginning of each definitive test using ACN extraction and U.S. EPA Method 8330A (USEPA, 2007). Samples for chemical analysis were hydrated for 24 h in

accordance with Soil Amendment Procedures (Section 2.3) prior to extraction. Soil dry-fraction (dry weight/wet weight) was determined in triplicate from subsamples of each treatment concentration. For extraction, 2 g treatment and control samples were collected from each soil batch and placed into respective 50 mL polypropylene centrifuge tubes, and 10 mL of ACN was added to each tube. Samples were vortexed with ACN for 1 min, then sonicated in darkness for 18 h at 20 °C. After the sonicated samples had settled for 1 h at room temperature, 5 mL of each supernatant was transferred into glass tubes that contained 5 mL of $CaCl_2$ solution (5 g L^{-1}) as a flocculent. Supernatant was filtered through a 0.45 µm polytetrafluoroethylene (PTFE) syringe cartridge, and 1 mL of each filtered solution was transferred into an HPLC vial. Soil extracts were analyzed and quantified by HPLC.

2.6 Adapted Toxicity Characteristic Leaching Procedure (ATCLP): Extraction of TNT from Soil

In addition to extraction with ACN, TNT was extracted from soil using the ATCLP (Haley et al., 1993) at the beginning of each definitive test. The ATCLP is a modification of the toxicity characteristic leaching procedure (TCLP) (40 CFR Part 268.41, Hazardous Waste Management, Method 1311). The procedure was modified by substituting CO_2-saturated water for acetic acid to acidify the water used for extraction and thereby simulate the soil–water conditions that exist due to respiration by soil biota. To retain the effects of the natural buffering capacity of the soil, the CO_2-saturated water was not recharged once it had been added to the soil (unlike the acetic acid in the TCLP). All extractions were performed in triplicate. For each treatment concentration, 4 g of soil were transferred into 20 mL vials, and 16 mL of CO_2-saturated water (pH 3.8 to 4.0) was added. The vials were immediately sealed. Soil samples were vortexed for 45 s then mixed in darkness for 18 h on a rotary end-over-end mixer (30 rpm) at room temperature (40 CFR Part 268.41). The mixture was allowed to settle for at least 2 h before supernatants were filtered through 0.45 µm PTFE syringe cartridges. An equivalent volume of ACN was added to each filtered soil extract before the HPLC analysis was performed.

ATCLP-based extractions were not conducted in studies with RDX because all concentrations selected for toxicity tests with *E. crypticus* exceeded the aqueous solubility of RDX (42 mg L^{-1} at 20 °C; Monteil-Rivera et al., 2004).

2.7 Analytical Determinations of TNT and RDX

Soil extracts were analyzed and EM concentrations were quantified by reversed-phase HPLC using a modified U.S. EPA Method 8330A. The method was modified by adjusting the flow rate of the 50:50 methanol–water mobile phase to 1.0 mL min^{-1} rather than 1.5 mL min^{-1}. A 25 cm × 4.6 mm × 5 µm particle size C-18 column was used for all determinations. For HPLC, Beckman System Gold analytical instrumentation (Beckman Coulter, Inc., Brea, CA) was used, which consists of a model 126 programmable solvent module, model 168 diode array detector, and a model 507 automatic sampler. Calibration curves were generated before each HPLC run by dissolving certified standards (AccuStandard, Inc.) of each EM in a 50:50 water–ACN solution in a range of concentrations appropriate for each run. The method detection limit was 0.05 mg L^{-1} in solution and 0.5 mg kg^{-1} in soil. In addition, solution

reagent blanks and standards were placed intermittently between samples. All chemical concentrations in soil were expressed on dry mass basis. Nominal and analytically determined (measured) concentrations used in the definitive tests are shown in Tables 2–13.

2.8 Toxicity Assessments

Several soil invertebrate toxicity tests, for which standardized protocols have been developed by the International Organization for Standardization (ISO, 1998a, 1998b), can effectively be used to assess toxicity and derive protective benchmark values for EMs (Stephenson et al., 2002; Løkke and Van Gestel, 1998). We adapted the ISO 16387 bioassay, *Soil Quality: Effects of Pollutants on Enchytraeidae (*Enchytraeus *sp)—Determination of Effects on Reproduction and Survival* (ISO, 2004) to assess the effects of TNT or RDX on the enchytraeid worm *E. crypticus*. This test was selected on the basis of its use measuring chemical toxicity to ecologically relevant test species during chronic assays, and also because of its inclusion of at least one reproduction component among the measurement endpoints. The ISO guideline for this assay was originally developed for use with Organisation for Economic Co-operation and Development (OECD, 1984) artificial soil (similar soil formulation was later adapted for U.S. EPA standard artificial soil [SAS]; USEPA, 1996; and for ASTM artificial soil [AS]; ASTM E1676-04, 2004b). However, several studies demonstrated that this test could also be conducted using natural soils (Amorim et al., 2005a, 2005b, 2009; Kuperman et al., 2003, 2004a, 2004b, 2005, 2006a–2006d). The ISO 16387 bioassay was initially developed using the enchytraeid worm species *Enchytraeus albidus*. Results of our previous studies using *E. albidus* showed that for optimal test conditions, this species requires soils containing high organic matter (OM) content with pH 6 (±0.5). *E. albidus* performed poorly in natural soils having physical and chemical characteristics that support a higher level of EM bioavailability (Amorim et al., 2005a, 2005b, 2009; Kuperman et al., 1999, 2006a). *E. crypticus*, which is listed in the ISO protocol as an acceptable alternative to *E. albidus*, was therefore selected for toxicity testing.

Potworms were bred in 4.3 L clear plastic boxes (34 × 20 × 10 cm) filled with 2 kg (dry mass) moist SSL soil. The culture was kept in an environment-controlled incubator under a 16 h light–8 h dark photoperiod cycle with a mean photosynthetically active radiation (PAR) light intensity of 12.8 ± 0.7 µM m^{-2} s^{-1} (985 ± 52 lux) and mean temperature of 21.6 ± 0.1 C. Soil moisture level was adjusted to 100% of the WHC of SSL soil and was maintained by periodic (once per week) mass checks and water adjustments. Soil in the breeding culture was aerated by carefully mixing it once each week. The potworms were fed approximately twice each week with ground oats spread onto the soil surface. If food from the previous feeding date remained on the soil surface, the amount of food added was adjusted. Every 6 weeks, the worms were transferred into a freshly prepared culture substrate. Cultures were synchronized so that all worms used in each toxicity test were approximately the same age. The potworm culture was deemed healthy if worms were whitish in color, reproduced continuously, did not try to leave the soil, and exhibited a shiny outer surface with no soil particles clinging to them.

Glass vessels (jars; 42 mm i.d. × 45 mm height) were used as test containers. Before the jars were used in the toxicity tests, they were cleaned with acetone, rinsed successively with tap water and ASTM Type I water, then air-dried. Twenty grams (dry mass basis) of prepared treatment soil and 0.05 g of ground oats were added to each test container,

which was then mixed and hydrated to 100% of the WHC of each soil. The mass of each container with hydrated soil was recorded.

Adult potworms with eggs in the clitellum region were used for testing. They were collected from culture and placed in a Petri dish filled with a small amount of ASTM Type I water for examination with a stereomicroscope. Potworms with no eggs were discarded; any invertebrates living in the cultures (such as mites) were also removed. Ten potworms, selected for uniformity (approximately 1 cm in length), were placed on top of the prepared hydrated treatment soil in each test container. Transparent plastic wrap was stretched over the top of each container and secured with a rubber band. Three pinholes were made in the plastic wrap to facilitate air exchange. All test containers were placed in an environment-controlled incubator under a 16 h light–8 h dark photoperiod cycle with a mean PAR light intensity of 12.8 ± 0.7 $\mu M\ m^{-2}\ sec^{-1}$ (985 ± 52 lux) and a mean temperature of 21.6 ± 0.1 °C for the duration of the 28 day test. The containers were weighed once each week, and the mass loss was replenished with the appropriate amount of ASTM Type I water. At that time, ground oats (0.05 g) were added atop the soil within each test container.

After 14 days, soil in each test container was carefully searched, and adult potworms were removed and counted. Potworms were examined for any morphological or behavioral changes. The remaining test substrate, including any cocoons laid during the first 2 weeks of the test, was incubated for additional 14 days. After 28 days from the start of the test, soil in the test containers was fixed with 70% ethanol, and 9 drops of Rose Bengal biological stain (1% solution in ethanol) were added. Staining continued for at least 24 h. The content of each test container was wet-sieved using a no. 100 (150 µm) mesh sieve, and retained contents were transferred to a counting tray where potworms were counted. Measurement endpoints included the number of adults surviving after 14 days and number of juveniles produced after 28 days.

Treatment concentrations for each definitive test with TNT and for the definitive tests with RDX in TSL or SSL soils were selected based on the results of the range-finding tests, which were performed to bracket the 20 and 50% inhibition in juvenile production compared with juvenile production in carrier controls for each soil. Based on the range-finding test results, limit tests were conducted to assess the effects of RDX W-A in KCL, RCL, and WCL soils on adult survival and production of juveniles by *E. crypticus*. The limit test is a definitive test variant to statistically compare treatment effects between the carrier (acetone) control treatment and the single greatest treatment concentration. It is performed when statistical analysis of the range-finding test data shows no significant differences among all treatment concentrations of a test chemical. Definitive tests included negative control (no chemicals added), carrier (acetone) control, positive control, and SSL soil control (SSL hydrated with ASTM Type I water to 100% of the WHC). SSL soil control was included only in studies with TSL, KCL, RCL, and WCL. The positive control was prepared as a solution of beryllium sulfate in ASTM Type I water added to SSL to obtain nominal beryllium concentration of 45 mg kg^{-1}. Performance of an *E. crypticus* culture was deemed acceptable if juvenile production in the positive control remained within the range of 30–50% of juvenile production in the SSL control. The following replication was used in the definitive tests: four replicates of each EM treatment and controls for definitive tests with multiple treatment concentrations; in the limit tests, eight replicates in the

0 mg kg^{-1} (carrier control) and the 10,000 mg kg^{-1} (the greatest nominal RDX concentration, selected for studies with KCL, RCL, and WCL soils) treatments; and four replicates in the negative, positive, and SSL soil controls. Validity criteria for the negative controls are included the following performance parameters (ISO, 2004):

- The adult mortality does not exceed 20% after 14 days;
- The average number of juvenile potworms per test container at the end of the test is greater than 2.5-fold the initial number of adult potworms per test container; and
- The coefficient of variation for the mean number of juveniles is ≤50%.

2.9 Data Analyses

Adult survival and juvenile production data were analyzed using regression models selected from among those described in an Environment Canada guidance document (Environment Canada, 2005). During the model selection process, compliance with the normality assumptions and homoscedasticity of the residuals were determined by examining the stem-and-leaf graphs and histograms of the residuals. The best fit was evident when the regression lines generated by the models were closest to the data points; the regression coefficients for point estimates were the greatest; the residuals were homoscedastic (i.e., had most random scattering); and the means, standard errors, and variances of the residuals were the smallest. The models selected for data analyses in these studies were logistic (Gompertz; eq 1) or logistic hormetic (eq 2). The logistic hormetic model has an additional parameter to accommodate hormesis, an effect whereby low concentrations of the test chemical stimulate the performance of the test organisms compared to the control organisms:

$$Y = a \times e^{\{[\log(1-p)]\times(\mathrm{C}\div\mathrm{ECp})^{b}\}} \quad (1)$$

$$Y = \frac{a\times[1+(h\times\mathrm{C})]}{1+[(p+(h\times\mathrm{C}))\div(1-p)]\times[\mathrm{C}\div\mathrm{ECp}]^{b}} \quad (2)$$

where

- Y is the dependent variable for a measurement endpoint (e.g., number of juveniles or adults);
- a is the y-axis intercept (i.e., the control response);
- e is the exponent of the base of the natural logarithm;
- p is the desired value for "p" effect (e.g., 0.50 for a 50% decrease from the control response; EC_{50});
- C is the exposure concentration in test soil;
- ECp is the estimate of effect concentration for a specified percent effect;
- h is the hormetic effect parameter; and
- b is a scale parameter that defines the shape of the equation.

The ECp parameters used in these studies included the TNT or RDX concentration producing a 20% (EC_{20}) or 50% (EC_{50}) decrease in the measurement endpoint compared with the carrier control. The EC_{50} parameter, a commonly reported value, was

included to enable comparisons of the results produced in these studies with results reported by other researchers. The 95% confidence intervals (CIs) associated with the point estimates were determined.

Analysis of variance was used to determine the bounded (when possible) no-observed-effect concentration (NOEC) and the lowest-observed-effect-concentration (LOEC) values for adult survival and juvenile production data, respectively. When no-observed-adverse-effect concentration (NOAEC) or lowest-observed-adverse-effect concentration (LOAEC) values were determined, the same statistical methods were used. Mean separations were done using Fisher's least-significant difference (FLSD) pairwise comparison tests. Student's *t*-test (two tailed) was used for pairwise comparisons in the limit test. The relationships among the selected soil parameters and toxicity data were determined using Pearson's correlation analysis followed by curve fitting for the highly correlated EC_{50}/soil property pairs using appropriate regression models. All analyses were performed using untransformed data and analytically determined TNT or RDX concentrations. A significance level of $p \leq 0.05$ (95% confidence level) was accepted for all statistical tests. Statistical analyses were performed using SYSTAT 11.0 (Systat Software, Inc., Chicago, IL) or SigmaPlot 10.0 (Systat Software, Inc., San Jose, CA).

3. RESULTS

3.1 Analytical Determinations of TNT in Soil

Concentrations of TNT within amended soil were determined at the beginning of each definitive toxicity test using both ACN-based and ATCLP-based extractions. This report presents the results of analytical determinations of TNT concentrations in TSL, KCL, RCL, and WCL soils. Data established in studies with SSL soil were reported in Kuperman et al. (2006e) and are summarized in Tables 4 and 5 for readers' convenience. Samples prepared for weathering-and-aging of TNT in test soils were analyzed to determine the initial TNT concentrations. These concentrations were then contrasted with TNT concentrations at the end of the weathering-and-aging procedure to assess the net effect of weathering-and-aging of TNT in soil on the exposure conditions for *E. crypticus* during respective toxicity tests and to determine TNT FA versus W-A in soil. Analytically determined initial concentrations were also used for monitoring ACN-extractable TNT concentrations during the weathering-and-aging process to determine the time when TNT concentrations were effectively stabilized and/or had declined to ≤5% of the initial concentration.

The weathering-and-aging procedures performed in this study revealed differential rates of decreases in soil TNT concentrations over time according to soil type. Concentrations of TNT decreased more rapidly over time in the three clay loam soils (RCL, KCL, and WCL) than in the two sandy loam soils (TSL and SSL). Decreases in the analytically determined TNT concentrations in the nominal 100 mg kg^{-1} treatment level in the five soils are shown in Figure 1. These changes in soil TNT concentrations were typical of other nominal TNT treatments measured periodically over the 3 month weathering-and-aging procedures and are shown in Figure 1 as examples because the nominal 100 mg kg^{-1} was the only treatment level that was selected for use in all five soils tested in this study. Based on the results for each

TNT–soil pairing, day 82, from the initial hydration of each test soil to 60% of the WHC, was designated for terminating the weathering-and-aging procedures and for commencement of definitive toxicity testing with *E. crypticus* in each of the five soils.

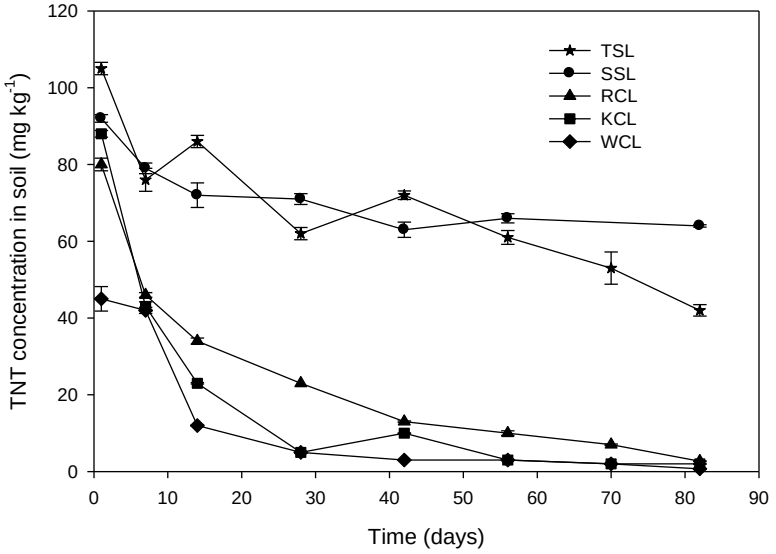

Figure 1. Concentrations of TNT in soil analytically determined during the 3-month weathering-and-aging process. Concentration values are means and standard errors (SEs; n = 3). Natural soils TSL, SSL, KCL, RCL, and WCL were initially amended with 100 mg kg^{-1} nominal TNT concentration. Initial concentration was determined after a 24 h moisture equilibration of FA soils hydrated to 60% of the WHC of each soil.

3.1.1 TNT Concentration in TSL Soil

Mean values for ACN-extractable TNT within FA TSL soil, expressed as percentage of amendment, ranged from 40% at nominal 10 mg kg^{-1} to 91% at nominal 120 mg kg^{-1} (Table 2). This indicates that a portion of TNT was rapidly transformed/degraded, strongly sorbed onto soil, or a combination of these processes during the initial 24 h period after soil hydration. Mean concentrations of ATCLP-extractable TNT within FA TSL soil ranged from 25 to 60% of the mean concentrations of ACN-extractable TNT (Table 2).

Table 2. Concentrations of TNT FA into TSL Soil Used in Definitive Toxicity Tests with *E. crypticus*

Nominal Concentration (mg kg^{-1})	ACN Extraction (mg kg^{-1})	SE	ACN/ Nominal (%)	ATCLP Extraction (mg kg^{-1})	SE	ATCLP/ ACN (%)
0	BDL	BDL	NA	BDL	BDL	NA
10	4	0.1	40	1	0.1	25
60	49	1.0	82	24	0.1	49
80	69	0.7	86	37	0.1	54
100	88	1.3	88	50	0.5	57
120	109	1.6	91	65	1.4	60

Note: Analytically determined concentrations (means and SEs; $n = 3$) are based on ACN extraction (U.S. EPA Method 8330A) or water extraction (ATCLP) of TNT from soil.
NA, not applicable.
BDL, below detection limit (0.05 mg L^{-1} in solution and 0.5 mg kg^{-1} in soil).

Mean values for TNT W-A in TSL soil and remaining ACN-extractable, calculated as percentages of corresponding initial concentrations of ACN-extractable TNT in FA soils, ranged from 15% (at nominal 20 mg kg^{-1}) to 78% (at nominal 350 mg kg^{-1}) of initial values (Table 3). The greatest percentage decrease (85%) occurred in the lowest nominal TNT treatments, nominal 20 mg kg^{-1}. The percentage decrease in ACN-extractable TNT during the weathering-and-aging procedure was lower and more uniform at greater nominal concentrations of 100–350 mg kg^{-1} (Table 3). Mean concentrations of ATCLP-extractable TNT W-A in TSL soil ranged from 22 to 60% of corresponding ACN-extractable TNT concentrations (Table 3). Overall, mean ratios of ATCLP-extractable TNT versus ACN-extractable TNT remained relatively steady across treatment levels within TSL soil, with an average of 49% in FA soil and 50% when TNT was W-A in TSL soil. These TSL soils, wherein TNT was W-A, were the soil treatments used in definitive toxicity tests.

Table 3. Concentrations of TNT W-A in TSL Soil Used in Definitive Toxicity Tests with *E. crypticus*

Nominal Concentration (mg kg^{-1})	Initial ACN (mg kg^{-1})	W-A ACN (mg kg^{-1})	W-A/ Initial ACN (%)	W-A ATCLP (mg kg^{-1})	W-A ATCLP/ W-A ACN (%)
0	BDL	BDL	NA	BDL	NA
20	17 (4)	2.5 (0.1)	15	0.56 (0.01)	22
50	52 (5)	27 (0.7)	52	11 (0.03)	41
100	54 (4)	42 (1.5)	78	21 (0.4)	50
160	181 (13)	135 (2)	75	76 (0.5)	56
200	229 (14)	175 (2)	76	96 (0.9)	55
225	268 (15)	191 (3)	71	108 (0.3)	57
275	328 (20)	233 (2)	71	133 (1)	57
350	370 (14)	289 (2)	78	172 (1)	60

Note: Analytically determined concentrations, shown as means and SEs (parentheses), $n = 3$, were based on ACN extraction (U.S. EPA Method 8330A) or water extraction (ATCLP) of TNT from soil.

3.1.2 TNT Concentration in SSL Soil

Mean values for ACN-extractable TNT within FA SSL soil averaged 88% (ranging from 80 to 96%) of nominal concentrations (Table 4). Mean values for ATCLP-extractable TNT within FA SSL soil treatments, expressed as percentages of the concentrations of ACN-extractable TNT within corresponding treatments, averaged 70% (ranging from 63 to 75%) of ACN-extractable TNT (Table 4).

Table 4. Concentrations of TNT FA into SSL Soil Used in Definitive Toxicity Tests with *E. crypticus*[*]

Nominal Concentration (mg kg^{-1})	ACN Extraction (mg kg^{-1})	SE	ACN/ Nominal (%)	ATCLP Extraction (mg kg^{-1})	SE	ATCLP/ACN (%)
0	BDL	BDL	NA	BDL	BDL	NA
50	40	0.4	80	25	0.2	63
75	62	2.1	82	40	0.5	67
100	85	0.2	85	59	1.2	69
150	134	6.1	90	98	0.6	73
200	186	2.4	93	137	0.9	73
300	287	2.5	96	215	1.0	75

[*]Modified from Kuperman et al. (2006e); included in this report for readers' convenience.
Note: Analytically determined concentrations (means and SEs; $n = 3$) are based on ACN extraction (U.S. EPA Method 8330A) or water extraction via ATCLP of TNT from soil.

Mean values for TNT W-A in SSL soil and remaining ACN-extractable, calculated as percentages of corresponding initial concentrations of ACN-extractable TNT in FA soils, ranged from 17% (at nominal 20 mg kg^{-1}) to 78% (at nominal 180 mg kg^{-1}) of initial values (Table 5). The greatest percentage decrease (83%) in ACN-extractable TNT within SSL following weathering-and-aging of TNT in soil occurred in the lowest nominal TNT treatment tested, 20 mg kg^{-1} in SSL. The percentage decrease in ACN-extractable TNT within SSL during the weathering-and-aging procedures was lower and more uniform at the greater nominal concentrations, 75–180 mg kg^{-1} (Table 5).

Mean values for ATCLP-extractable TNT W-A in SSL soil, expressed as percentages of the concentrations of ACN-extractable TNT within corresponding treatments, ranged from 13% (at nominal 20 mg kg^{-1}) to 33% (at nominal 150 mg kg^{-1}) of ACN-extractable TNT (Table 5). Mean ratios of ATCLP-extractable TNT versus ACN-extractable TNT decreased from an average of 70% within FA SSL soil to an average of 28% when TNT was W-A in SSL soil treatments.

Table 5. Concentrations of TNT W-A in SSL Soil Used in Definitive Toxicity Tests with *E. crypticus*[*]

Nominal Concentration (mg kg^{-1})	Initial ACN (mg kg^{-1})	W-A ACN (mg kg^{-1})	W-A/ Initial ACN (%)	W-A ATCLP (mg kg^{-1})	W-A ATCLP/ W-A ACN (%)
0	BDL	BDL	NA	BDL	NA
20	18 (0.2)	3 (0.5)	17	0.4 (0.01)	13
75	73 (0.8)	46 (0.7)	63	14 (0.1)	31
100	92 (0.8)	66 (2.5)	72	20 (0.3)	31
150	139 (5.3)	94 (0.0)	68	30.8 (0.36)	33
160	150 (0.6)	105 (2.7)	70	31.0 (1.43)	30
180	175 (8.6)	137 (3.8)	78	42 (1.3)	31

[*]Modified from Kuperman et al. (2006e); included in this report for readers' convenience. Analytically determined concentrations (means and SEs, $n = 3$) are based on ACN extraction (U.S. EPA Method 8330A) or water extraction via ATCLP of TNT from soil.

3.1.3 TNT Concentration in KCL Soil

Mean values for ACN-extractable TNT within FA KCL soil, expressed as percentage of amendment, averaged 95% (ranging from 82 to 108%) of nominal concentrations (Table 6). Mean values for ATCLP-extractable TNT within FA KCL soil treatments, expressed as percentages of the concentrations of ACN-extractable TNT within corresponding treatments, averaged 56% (ranging from 44 to 65%) of ACN-extractable concentrations (Table 6).

Table 6. Concentrations of TNT FA into KCL Soil Used in Definitive Toxicity Tests with *E. crypticus*

Nominal Concentration (mg kg^{-1})	ACN Extraction (mg kg^{-1})	SE	ACN/ Nominal (%)	ATCLP Extraction (mg kg^{-1})	SE	ATCLP/ ACN (%)
0	BDL	BDL	NA	BDL	BDL	NA
50	41	0.3	82	18	0.2	44
100	88	1	88	45	2	51
150	132	4	88	75	2	57
200	180	7	90	112	1	62
250	224	9	90	125	10	56
300	300	10	100	194	3	65
350	380	24	108	235	2	62
500	527	15	105	331	6	63
800	840	20	105	394	4	47

Note: Analytically determined concentrations (means and SEs; $n = 3$) are based on ACN extraction (U.S. EPA Method 8330A) or water extraction (ATCLP) of TNT from soil.

Mean values for TNT W-A in KCL soil and remaining ACN-extractable TNT, calculated as percentages of corresponding initial concentrations of ACN-extractable TNT in FA soils, ranged from 2% (at nominal 100 mg kg^{-1}) to 66% (at nominal 800 mg kg^{-1}) of the initial values (Table 7). Recovery of ACN-extractable TNT after weathering-and-aging in KCL was, in general, directly proportional to TNT concentrations (Table 7). The lowest percentage decrease (2%) in ACN-extractable TNT within KCL following weathering-and-aging of TNT in soil occurred in the lowest nominal TNT treatment tested, 100 mg kg^{-1} in KCL. The percentage decrease in ACN-extractable TNT within KCL during the weathering-and-aging procedures was lower and more uniform at greater nominal concentrations, 300–800 mg kg^{-1} (Table 7).

Mean values for ATCLP-extractable TNT W-A in KCL soil, expressed as percentages of the concentrations of ACN-extractable TNT within corresponding treatments, ranged from 25% (at nominal 100 mg kg^{-1}) to 55% (at nominal 500 mg kg^{-1}) of ACN-extractable TNT (Table 7). Mean ratios of ATCLP-extractable TNT versus ACN-extractable TNT decreased from an average of 56% within FA KCL soil to an average of 41% when TNT was W-A in KCL soil treatments.

Table 7. Concentrations of TNT W-A in KCL Soil Used in Definitive Toxicity Tests with *E. crypticus*

Nominal Concentration (mg kg^{-1})	Initial ACN (mg kg^{-1})	W-A ACN (mg kg^{-1})	W-A/ Initial ACN (%)	W-A ATCLP (mg kg^{-1})	W-A ATCLP/ W-A ACN (%)
0	BDL	BDL	NA	BDL	NA
100	88 (1)	2 (0.1)	2	0.5 (0.02)	25
150	132 (4)	12 (0.3)	9	4.2 (0.06)	35
250	224 (9)	26 (6.5)	12	6.5 (1.5)	25
300	300 (10)	147 (1.6)	49	71 (0.8)	48
350	380 (24)	147 (4.3)	39	65 (0.7)	44
500	527 (15)	313 (7.7)	59	172 (0.3)	55
800	840 (20)	553 (6.9)	66	297 (2.1)	54

Note: Analytically determined concentrations, shown as means and SEs (parentheses), $n = 3$, were based on ACN extraction (U.S. EPA Method 8330A) or water extraction (ATCLP) of TNT from soil.

3.1.4 TNT Concentration in RCL Soil

Mean percentages of ACN-extractable TNT within FA RCL soil averaged 86% (ranging from 80 to 90%) of nominal concentrations (Table 8). Mean values for ATCLP-extractable TNT within FA RCL soil treatments, expressed as percentages of the concentrations of ACN-extractable TNT within corresponding treatments, averaged 48% (ranging from 33 to 59%) of ACN-extractable concentrations (Table 8).

Table 8. Concentrations of TNT FA into RCL Soil Used in Definitive Toxicity Tests with *E. crypticus*

Nominal Concentration (mg kg^{-1})	ACN Extraction (mg kg^{-1})	SE	ACN/ Nominal (%)	ATCLP Extraction (mg kg^{-1})	SE	ATCLP/ ACN (%)
0	BDL	BDL	NA	BDL	BDL	NA
100	80	2	80	34	1	42
200	168	2	84	85	2	51
300	267	6	89	152	3	57
400	330	2	83	194	3	59
600	538	6	90	262	2	49
1000	900	13	90	297	3	33

Note: Analytically determined concentrations (means and SEs; $n = 3$) are based on ACN extraction (U.S. EPA Method 8330A) or water extraction (ATCLP) of TNT from soil.

Mean values for TNT W-A in RCL soil that remained ACN-extractable, calculated as percentages of corresponding initial concentrations of ACN-extractable TNT in FA soils, ranged from 13% (at nominal 300 mg kg^{-1}) to 53% (at nominal 700 mg kg^{-1}) of initial values (Table 9). Mean values for ATCLP-extractable TNT W-A in RCL soil, expressed as percentages of the concentrations of ACN-extractable TNT within corresponding treatments, ranged from 40 to 57% of ACN-extractable concentrations (Table 9). Overall, mean ratios of ATCLP-extractable TNT versus ACN-extractable TNT were similar when the TNT was both FA and W-A in RCL soil treatments (48 and 49%, respectively).

Table 9. Concentrations of TNT W-A in RCL Soil Used in Definitive Toxicity Tests with *E. crypticus*

Nominal Concentration (mg kg^{-1})	Initial ACN (mg kg^{-1})	W-A ACN (mg kg^{-1})	W-A/ Initial ACN (%)	W-A ATCLP (mg kg^{-1})	W-A ATCLP/ W-A ACN (%)
0	BDL	BDL	NA	BDL	NA
300	267 (6)	35 (1)	13	14 (0.5)	40
600	538 (6)	93 (2)	17	43 (0.3)	46
700	268 (8)	142 (4)	53	72 (0.5)	51
800	708 (24)	253 (3)	36	135 (0.3)	53
1000	900 (13)	391 (8)	43	224 (2.0)	57

Note: Analytically determined concentrations (means and SEs; $n = 3$) are based on ACN extraction (U.S. EPA Method 8330A, ACN) or water extraction (ATCLP) of TNT from soil.

3.1.5 TNT Concentration in WCL Soil

Mean values for ACN-extractable TNT within FA WCL soil, expressed as percentage of amendment, averaged 93% (ranging from 57 to 104%) of nominal concentrations (Table 10). Mean values for ATCLP-extractable TNT within FA WCL soil, expressed as percentages of concentrations of ACN-extractable TNT within corresponding treatments, ranged from 13 to 53% of ACN-extractable concentrations (Table 10).

Table 10. Concentrations of TNT FA into WCL Soil Used in Definitive Toxicity Tests with *E. crypticus*

Nominal Concentration (mg kg^{-1})	ACN Extraction (mg kg^{-1})	SE	ACN/ Nominal (%)	ATCLP Extraction (mg kg^{-1})	SE	ATCLP/ ACN (%)
0	BDL	BDL	NA	BDL	BDL	NA
150	86	9	57	11	1	13
250	245	11	98	83	1	34
300	284	17	95	100	4	35
400	387	6	97	159	1	41
450	433	7	96	178	6	41
600	564	21	94	272	2	48
700	670	30	96	336	7	50
800	835	17	104	390	16	47
900	890	46	99	474	12	53

Note: Analytically determined concentrations (means and SEs; n = 3) are based on ACN extraction (U.S. EPA Method 8330A) or water extraction (ATCLP) of TNT from soil.

Mean values for TNT W-A in WCL soil that remained ACN-extractable, calculated as percentages of corresponding initial concentrations of ACN-extractable TNT in FA soils, ranged from 8% (at nominal 150 mg kg^{-1}) to 79% (at nominal 700 mg kg^{-1}) of initial values (Table 11). Mean values for ATCLP-extractable TNT W-A in WCL soil, expressed as percentages of the concentrations of ACN-extractable TNT within corresponding treatments, ranged from 16 to 39% of ACN-extractable concentrations (Table 11). Overall, mean ratios of ATCLP-extractable TNT versus ACN-extractable TNT decreased from an average of 40% within FA WCL soil to an average of 25% when TNT was W-A in WCL soil treatments.

Table 11. Concentrations of TNT W-A in WCL Soil Used in Definitive Toxicity Tests with *E. crypticus*

Nominal Concentration (mg kg^{-1})	Initial ACN (mg kg^{-1})	W-A ACN (mg kg^{-1})	W-A /Initial ACN (%)	W-A ATCLP (mg kg^{-1})	W-A ATCLP/ W-A ACN (%)
0	BDL	BDL	NA	BDL	NA
150	86 (9)	7 (0.3)	8	0	0
300	284 (17)	30 (0.3)	11	5 (0.6)	16
500	522 (23)	165 (2.3)	32	45 (0.2)	27
600	564 (21)	182 (1.9)	32	58 (0.3)	32
700	670 (30)	528 (25)	79	179 (0.5)	34
900	890 (46)	695 (11)	78	272 (1.6)	39

Note: Analytically determined concentrations, shown as means and SEs (parentheses), n = 3, were based on ACN extraction (U.S. EPA Method 8330A) or water extraction (ATCLP) of TNT from soil.

3.2 Analytical Determinations of RDX in Soil

Definitive toxicity tests with multiple RDX concentrations were conducted separately with TSL and SSL soils. Based on the results of range-finding tests, limit tests were conducted to assess the effects of RDX on *E. crypticus* in KCL, RCL, and WCL soils. Toxicity tests for all soils included *E. crypticus* exposures to RDX FA and W-A in soil. Concentrations of RDX in amended soils were determined at the beginning of each definitive toxicity test using ACN extractions and U.S. EPA Method 8330A (USEPA, 2007). ATCLP-based extractions were excluded from studies with RDX because all concentrations selected for toxicity tests with *E. crypticus* greatly exceeded the aqueous solubility of RDX (42 mg L^{-1} at 20 °C; Monteil-Rivera et al., 2004).

This report presents the results of both RDX amendments into soils and those from analytical determinations of RDX concentrations in TSL, KCL, RCL, and WCL soils. Nominal and ACN-extractable concentrations of RDX in TSL soil are shown in Table 12. Data established in studies with SSL soil were reported in Kuperman et al. (2004a) and are summarized in Table 13 for convenience of comparison with other soils. Average analytically determined concentrations (n = 3; SEs in parentheses) in nominal 10,000 mg kg^{-1} treatments of RDX FA and W-A in soil, respectively, were: 12,300 (250) and 9840 (590) mg kg^{-1} in KCL; 10,600 (830) and 9720 (480) mg kg^{-1} in RCL; and 16,240 (1270) and 10,700 (350) mg kg^{-1} in WCL. Analytically determined concentrations of RDX in the nominal 10,000 mg kg^{-1} treatment were greater after the 3-month weathering-and-aging in TSL, compared with the analytically determined RDX concentrations in FA TSL. This likely resulted from either the soil amendment procedure used for this treatment level, which included the addition of dry, crystalline RDX to clean soil, or the unequal redistribution of RDX crystals within the soil during subsequent periodic manual mixing of soil batches after each soil rehydration cycle during the 3 month weathering-and-aging in a greenhouse, or from a combination of both.

Table 12. Concentrations of RDX FA and RDX W-A in TSL Soil Used in Definitive Toxicity Tests with *E. crypticus*

Nominal (mg kg^{-1})	RDX FA (mg kg^{-1})	SE	RDX W-A (mg kg^{-1})	SE
0	BDL	BDL	BDL	BDL
1,000	1,200	260	1,060	63
2,000	2,200	73	2,300	60
4,000	4,500	700	5,560	564
5,000	6,300	715	7,000	1,300
10,000	10,000	787	15,000	1,100

Note: Analytically determined concentrations (means and SEs; n = 3) are based on ACN extraction (U.S. EPA Method 8330A) of RDX from soil.

Table 13. Concentrations of RDX FA and RDX W-A in SSL Soil Used in Definitive Toxicity Tests with *E. crypticus*[*]

Nominal (mg kg^{-1})	RDX FA (mg kg^{-1})	SE	RDX W-A (mg kg^{-1})	SE
0	BDL	BDL	BDL	BDL
300	300	19	NT	NT
600	660	19	NT	NT
1,200	1,200	22	1,050	20
2,400	2,200	72	2,380	41
4,800	4,560	140	3,985	43
10,000	10,000	370	9,550	370
20,000	21,400	1,200	18,350	520

[*]Modified from Kuperman et al. (2004a); included in this report for convenience of comparison with other soils reported herein.
Note: Analytically determined concentrations (means and SEs; $n = 3$) are based on ACN extraction (U.S. EPA Method 8330A) of RDX from soil.
NT, not tested in the study.

3.3 Effects of TNT on the Potworm *E. crypticus*

Definitive studies using the enchytraeid toxicity test in ISO 16387 (2004) were conducted to assess the acute (adult mortality) and chronic (juvenile production) effects of TNT on the potworm *E. crypticus* in TSL, SSL, KCL, RCL, and WCL soils. Within each type of soil, adult potworms were exposed to a range of TNT concentrations in independent investigations. Measurement endpoints were assessed using treatment concentrations that were based on the results of the range-finding studies. Measurement endpoints included number of surviving adults after 14 days and number of juveniles produced after 28 days. Exposure concentrations for each soil were selected to achieve bracketing of significant effects on reproduction endpoints, i.e., production of juveniles. Reproduction endpoints are preferred for the development of Eco-SSL values for soil invertebrates (USEPA, 2005) and were therefore the main focus of these studies. The ranges of exposure concentrations were expanded to allow determination of the concentrations that caused lethal effects to adults. All ecotoxicological parameters were estimated using these measurement endpoint values and concentrations of TNT in soil that were analytically determined utilizing U.S. EPA Method 8330A (USEPA, 2007).

Test results complied with the validity criteria defined in the ISO 16387 (2004) test guideline and those stipulated in Section 2.9 of this report. Mean adult survival in negative controls ranged from 93 to 100% in tests with FA soil and from 95 to 100% in tests with TNT W-A in soil (Tables 14–18). The mean numbers of juveniles produced by *E. crypticus* in the negative controls of each test with TNT greatly exceeded the minimum requirement of 25 individuals (Tables 14–18). All coefficients of variation (CVs) for the numbers of juveniles produced by *E. crypticus* in negative controls were <50%, as required by the ISO 16387 test guideline: in studies with FA TNT, CVs were 6, 10, 7, 15, and 36% in TSL, SSL, KCL, RCL, and WCL soils, respectively; and in studies with TNT W-A in these soils, CVs were 32, 3, 24, 17, and 9% in TSL, SSL, KCL, RCL, and WCL, respectively. The mean numbers of juveniles in

positive controls of studies with FA TNT were 40, 44, 35, 33, and 36% of the values in the respective SSL soil control treatments in tests with TSL, SSL (negative control data were used), KCL, RCL, and WCL soils. The mean numbers of juveniles in positive controls for studies with TNT W-A in soil were 42, 41, 39, 31, and 34% of the values in the respective SSL soil control treatments in tests with TSL, SSL (negative control data were used), KCL, RCL, and WCL soils.

The performance of *E. crypticus* in positive controls, expressed as the mean percent decrease from juvenile production in SSL soil control treatments, was very consistent based on the mean, SE, and CV values of 37.6, 2.0, and 0.12%, respectively, in studies with FA TNT, and the values of 37.4, 2.1, and 0.13% for respective parameters determined in studies with TNT W-A in soil. Overall, the positive control data were consistent with the baseline established for the laboratory culture of *E. crypticus*. Direct comparisons of the results of positive control treatments are not possible because ISO 16387 was a relatively new method at the time these tests were conducted, and because ISO 16387 was originally optimized for testing chemicals with a different potworm species, *E. albidus*, in the OECD artificial soil. In addition, reference values for *E. crypticus* exposures in natural soils were not available from the literature. This issue was resolved later by a proposal to use boric acid as a universal reference toxicant and by establishing warning charts to continuously monitor the health and performance of test species maintained in ECBC laboratory cultures (Amorim et al., 2009; Kuperman et al., 2006a, 2008, 2009c). Compliance with the test validity criteria confirmed that the toxicological effects determined in the definitive tests were attributable to the TNT treatments.

Table 14. Adult Survival and Juvenile Production by *E. crypticus* Exposed to TNT FA or W-A in TSL Soil

TNT FA Treatment (mg kg^{-1})	Number of Adults	SE	Number of Juveniles	SE	TNT W-A Treatment (mg kg^{-1})	Number of Adults	SE	Number of Juveniles	SE
Negative*	9.8	0.3	260	8	Negative*	9.5	0.3	784	124
Acetone*	9.8	0.3	393	19	Acetone*	9.8	0.3	1766	80
Positive*	9.5	0.5	476	55	Positive*	8.8	0.5	544	51
SSL soil*	9.3	0.5	1202	62	SSL soil*	10	0.0	1309	8
2.5	NT	NT	NT	NT	2.5	9.8	0.3	1787	113
4	10	0.0	475	23	27	9.8	0.3	1988	21
49	9.5	0.3	525	40	42	10	0.0	795	69
69	9.8	0.3	299	80	135	7.3	1.0	5.5	3.5
88	9.0	0.4	177	27	175	5.3	0.9	0	0
109	6.5	1.0	66	30	191	7.0	1.5	0	0
233	NT	NT	NT	NT	233	4.0	1.9	0	0
289	NT	NT	NT	NT	289	4.8	1.4	0	0

*Respective control treatments.
Notes: Concentrations of TNT are based on ACN extraction (U.S. EPA Method 8330A).
Potworm numbers are means and SEs (n = 4).
NT, not tested in this study.

Table 15. Adult Survival and Juvenile Production by *E. crypticus* Exposed to TNT FA or W-A in SSL Soil[*]

TNT FA Treatment (mg kg^{-1})	Number of Adults	SE	Number of Juveniles	SE	TNT W-A Treatment (mg kg^{-1})	Number of Adults	SE	Number of Juveniles	SE
Negative[†]	9.3	0.5	1202	62	Negative[†]	10	0.0	1851	27
Acetone[†]	10	0.0	1029	73	Acetone[†]	10	0.0	2120	52
Positive[†]	9.5	0.5	532	84	Positive[†]	9.5	0.3	764	39
40	10	0.0	1273	49	3	9.8	0.3	2662	253
62	9.8	0.3	1013	72	46	9.5	0.5	1125	69
85	9.8	0.3	746	52	66	10	0.0	617	79
134	8.3	0.5	217	18	94	8.5	0.6	276	60
186	8.3	0.5	35	20	105	7.5	0.9	160	61
287	6.3	1.4	0	0	137	5.5	1.7	0	0

[*]Modified from Kuperman et al. (2006e).
[†]Respective control treatments.
Notes: TNT concentrations are based on ACN extraction (U.S. EPA Method 8330A). Potworm numbers are means and SEs (n = 4).

Table 16. Adult Survival and Juvenile Production by *E. crypticus* Exposed to TNT FA or W-A in KCL Soil

TNT FA Treatment (mg kg^{-1})	Number of Adults	SE	Number of Juveniles	SE	TNT W-A Treatment (mg kg^{-1})	Number of Adults	SE	Number of Juveniles	SE
Negative[*]	10	0.0	2317	86	Negative[*]	10	0.0	2323	280
Acetone[*]	10	0.0	2533	112	Acetone[*]	10	0.0	1973	91
Positive[*]	10	0.0	514	67	Positive[*]	8.8	0.5	766	62
SSL soil[*]	10	0.0	1464	74	SSL soil[*]	10	0.0	1973	144
41	9.8	0.3	1709	34	2	10	0.0	2065	92
88	9.8	0.3	1871	80	12	10	0.0	2255	81
132	10	0.0	1689	60	26	10	0.0	2007	133
180	10	0.0	1411	118	147.2	9.7	0.3	1589	321
224	9.0	0.4	657	86	147.4	10	0.0	1596	142
300	9.5	0.3	162	61	313	7.0	1.5	180	34
380	7.5	0.5	121	50	553	7.0	0.4	43	25
527	6.8	1.0	17	13	NT	NT	NT	NT	NT
840	4.0	2.0	0.0	0.0	NT	NT	NT	NT	NT

[*]Respective control treatments.
Notes: TNT concentrations are based on ACN extraction (U.S. EPA Method 8330A). Potworm numbers are means and SEs (n = 4).

Table 17. Adult Survival and Juvenile Production by *E. crypticus* Exposed to TNT FA or W-A in RCL Soil

TNT FA Treatment (mg kg^{-1})	Number of Adults	SE	Number of Juveniles	SE	TNT W-A Treatment (mg kg^{-1})	Number of Adults	SE	Number of Juveniles	SE
Negative*	10	0.0	1913	141	Negative*	10	0.0	1342	112
Acetone*	10	0.0	2203	185	Acetone*	10	0.0	2257	61
Positive*	9.3	0.8	454	36	Positive*	10	0.0	421	22
SSL soil*	10	0.0	1370	102	SSL soil*	9.8	0.3	1358	113
80	10	0.0	2411	101	35	10	0.0	2316	79
168	10	0.0	2292	170	93	10	0.0	2632	150
267	9.0	0.4	902	114	142	9.8	0.3	2264	155
330	7.5	0.5	453	115	253	4.3	1.6	251	109
538	7.3	0.5	197	28	391	0.0	0.0	0.0	0.0
900	6.5	0.9	55	25	NT	NT	NT	NT	NT

*Respective control treatments.

Notes: TNT concentrations are based on ACN extraction (U.S. EPA Method 8330A). Potworm numbers are means and SEs (n = 4).

Table 18. Adult Survival and Juvenile Production by *E. crypticus* Exposed to TNT FA or W-A in WCL Soil

TNT FA Treatment (mg kg^{-1})	Number of Adults	SE	Number of Juveniles	SE	TNT W-A Treatment (mg kg^{-1})	Number of Adults	SE	Number of Juveniles	SE
Negative*	9.5	0.3	2187	395	Negative*	9.8	0.3	2123	97
Acetone*	10	0.0	2252	85	Acetone*	10	0.0	2880	157
Positive*	9.0	1.0	583	32	Positive*	9.5	0.5	566	28
SSL soil*	9.8	0.3	1634	106	SSL soil*	10	0.0	1662	71
86	10	0.0	1984	64	7	10	0.0	2937	223
245	10	0.0	1888	66	30	10	0.0	2986	222
284	10	0.0	1641	58	165	9.8	0.3	2248	123
387	10	0.0	1309	234	182	10	0.0	1796	261
564	5.3	1.3	1117	290	528	9.8	0.3	931	111
670	7.8	0.6	431	77	695	7.5	0.3	29	14
835	8.0	1.1	6.3	3.0	NT	NT	NT	NT	NT
890	5.3	1.7	0.8	0.8	NT	NT	NT	NT	NT

*Respective control treatments.

Notes: TNT concentrations are based on ACN extraction (U.S. EPA Method 8330A). Potworm numbers are means and SEs (n = 4).

3.3.1 TNT Toxicity in TSL Soil

The ecotoxicological responses of *E. crypticus* to TNT FA and W-A in TSL soil are shown in Table 14. Both adult survival and juvenile production were affected in TNT-amended TSL soil within the concentration ranges selected for definitive tests (Table 14). Analytically determined acute ecotoxicological benchmarks for TNT are summarized in Table 19. For adult survival, the bounded NOEC and LOEC values based on ACN-extractable concentrations were 88 and 109 mg kg^{-1}, respectively, for TNT FA in TSL, and 42 and 135 mg kg^{-1}, respectively, for TNT W-A in TSL soil. Values based on ATCLP-extractable (aqueous) TNT concentrations were 50, 65, 76, and 96 mg kg^{-1} (Table 19).

The logistic Gompertz model had the best fit for the adult survival data. The EC_{20} and EC_{50} values for adult survival based on ACN-extractable concentrations were 101 and 117 mg kg^{-1}, respectively, for TNT FA in TSL, and 106 and 238 mg kg^{-1}, respectively, for TNT W-A in TSL soil. The EC_{20} and EC_{50} values for adult survival based on ATCLP-extractable concentrations were 59 and 71 mg kg^{-1}, respectively, for TNT FA in TSL, and 56 and 138 mg kg^{-1}, respectively, for TNT W-A in TSL soil (Table 19). Weathering-and-aging of TNT in TSL soil significantly decreased acute toxicity for *E. crypticus* based on the EC_{50} values and respective 95% CI (Table 19).

Table 19. Acute Ecotoxicological Benchmarks for TNT FA or W-A in TSL Soil Determined for Survival of Adult *E. crypticus*

Ecotoxicological Parameter	TNT FA (mg kg^{-1})	TNT W-A (mg kg^{-1})
	ACN Extraction	
NOEC	88	42
p	0.296	0.746
LOEC	109	135
p	<0.0001	0.004
EC_{20}	101	106
CI (95%)	92–110	27–185
EC_{50}	117	238*
CI (95%)	107–127	176–300
Model used	Gompertz	Gompertz
R^2	0.991	0.943
	ATCLP Extraction (Aqueous)	
NOEC	50	76
p	0.296	0.104
LOEC	65	96
p	<0.0001	0.011
EC_{20}	59	56
CI (95%)	53–65	9–102
EC_{50}	71	138*
CI (95%)	64–78	97–179
Model used	Gompertz	Gompertz
R^2	0.991	0.943

*Statistically significant (95% CI basis) decrease in toxicity following weathering-and-aging of TNT in soil.
Note: Concentrations are based on ACN extraction (U.S. EPA Method 8330A) or water extraction (ATCLP).
R^2, coefficient of determination.

The logistic model with a hormetic parameter (hormetic model) had the best fit for the data from toxicity tests with TNT FA in TSL soil, due to stimulation of juvenile production at the lower treatment concentrations (Figure 2). The EC_{20} and EC_{50} values for production of juveniles were 71 and 84 mg kg^{-1}, respectively, based on ACN-extractable TNT, and 39 and 47 mg kg^{-1}, respectively, based on ATCLP-extractable TNT (Table 20). The logistic Gompertz model had the best fit for the juvenile production data from toxicity tests with TNT W-A in TSL soil (Figure 2). The EC_{20} and EC_{50} values for production of juveniles were 26 and 41 mg kg^{-1}, respectively, based on ACN-extractable TNT, and 11 and 20 mg kg^{-1}, respectively, based on ATCLP-extractable TNT (Table 20). Weathering-and-aging of TNT in TSL soil significantly increased reproduction toxicity for *E. crypticus* based on either the EC_{20} or EC_{50} values and their respective 95% CIs (Table 20).

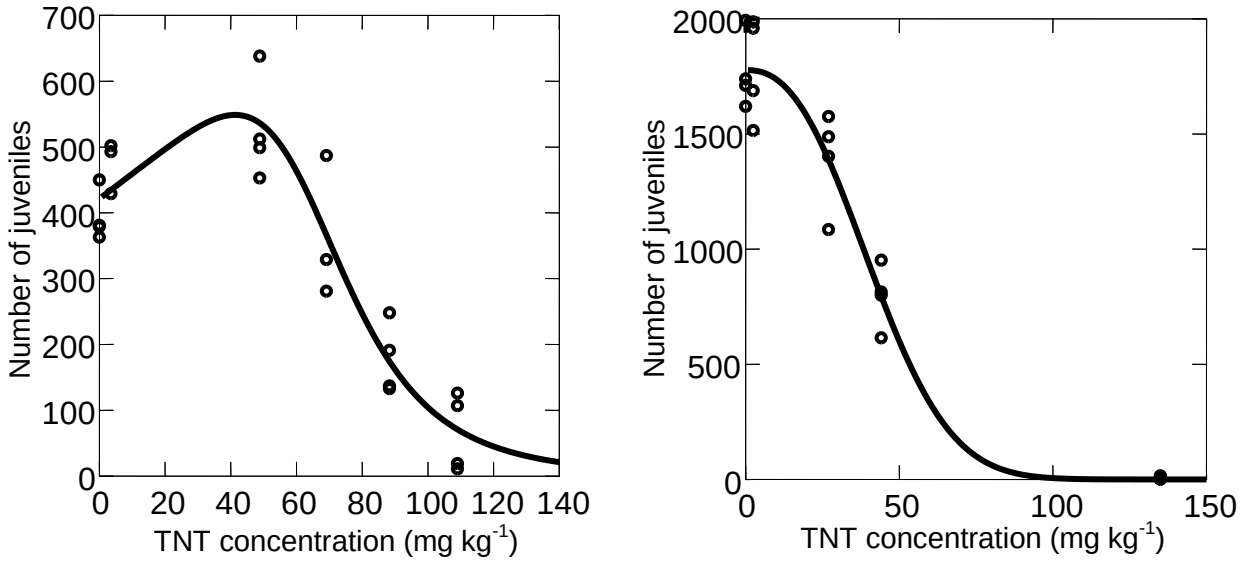

Figure 2. Effects of TNT FA (left) or W-A (right) in TSL soil on production of juveniles by *E. crypticus*.

Juvenile production was the more sensitive measurement endpoint for assessing TNT toxicity for *E. crypticus* in FA TSL compared with adult survival. Juvenile production was stimulated in the lowest two TNT concentrations of 4 and 49 mg kg^{-1} (Figure 2) in FA TSL, resulting in the respective 21 and 34% increases in the mean number of juveniles compared with carrier control. The increase at 49 mg kg^{-1} was statistically significant ($p = 0.012$) producing the bounded NOEC and LOEC values of 4 and 49 mg kg^{-1}, respectively, and the bounded NOAEC and LOAEC values of 69 and 88 mg kg^{-1}, respectively, based on ACN-extractable TNT. The bounded NOAEC and LOAEC values for juvenile production based on ATCLP-extractable TNT in FA TSL were 37 and 50 mg kg^{-1}, respectively (Table 20). The bounded NOEC and LOEC values for TNT W-A in TSL soil were 2.5 and 27 mg kg^{-1}, respectively, based on ACN-extractable TNT, and 0.56 and 11 mg kg^{-1}, respectively, based on ATCLP-extractable TNT (Table 20).

Coefficients of determinations (R^2) from nonlinear regression analyses of the reproduction toxicity data for TNT FA or TNT W-A in TSL soil, based on ACN-extractable or ATCLP-extractable TNT concentrations, were compared to determine which chemical measure of exposure better correlated with toxicity. These comparisons showed that coefficients were similar for both extraction types in either FA or W-A treatments (Table 20), which indicates that data from both extraction methods had excellent correlation with the toxicity data for juvenile production, and that neither method had an advantage in characterizing TNT bioavailability to *E. crypticus* in TSL soil.

Table 20. Chronic Ecotoxicological Benchmarks for TNT FA or W-A in TSL Soil Determined for Production of Juveniles by *E. crypticus*

Ecotoxicological Parameter	TNT FA (mg kg^{-1})	TNT W-A (mg kg^{-1})
	ACN Extraction	
NOEC	69	2.5
p	0.590	0.859
LOEC	88	27
p	<0.0001	0.006
EC_{20}	71	26*
CI (95%)	64–79	19–32
EC_{50}	84	41*
CI (95%)	77–91	37–46
Model used	Hormetic	Gompertz
R^2	0.974	0.988
	ATCLP Extraction (Aqueous)	
NOEC	37	0.56
p	0.590	0.850
LOEC	50	11
p	<0.0001	0.004
EC_{20}	39	11*
CI (95%)	34–44	7–14
EC_{50}	47	20*
CI (95%)	42–52	17–22
Model used	Hormetic	Gompertz
R^2	0.974	0.988

*Statistically significant (95% CI basis) increase in toxicity following weathering-and-aging of TNT in soil.
Note: Concentrations are based on ACN extraction (U.S. EPA Method 8330A) or water extraction (ATCLP).

3.3.2 TNT Toxicity in SSL Soil

Toxicity data for TNT established in our studies with SSL soil were reported previously in Kuperman et al. (2006e) and are included in Tables 15, 21, and 22 and Figure 2 of this report for convenience of comparison with other soils reported herein. For adult survival, the bounded NOEC and LOEC values based on ACN-extractable concentrations were 186 and 287 mg kg^{-1}, respectively, for TNT FA in SSL, and 94 and 105 mg kg^{-1}, respectively, for TNT W-A in SSL soil. Corresponding values based on ATCLP-extractable TNT concentrations were 137, 215, 30.8, and 31.0 mg kg^{-1} (Table 21).

Table 21. Acute Ecotoxicological Benchmarks for TNT FA or W-A in SSL Soil Determined for Survival of Adult *E. crypticus*[*]

Ecotoxicological Parameter	TNT FA (mg kg^{-1})	TNT W-A (mg kg^{-1})
	ACN Extraction	
NOEC	186	94
p	0.064	0.185
LOEC	287	105
p	<0.0001	0.032
EC_{20}	179	103
CI (95%)	100–258	82–124
EC_{50}	362	142[†]
CI (95%)	240–484	122–161
Model used	Gompertz	Gompertz
R^2	0.985	0.977
	ATCLP Extraction (Aqueous)	
NOEC	137	30.8
p	0.064	0.185
LOEC	215	31.0
p	<0.0001	0.032
EC_{20}	132	32[†]
CI (95%)	72–192	25–38
EC_{50}	275	44[†]
CI (95%)	177–372	38–50
Model used	Gompertz	Gompertz
R^2	0.985	0.977

[*]Modified from Kuperman et al. (2006e).
[†]Statistically significant (95% CI basis) increase in toxicity following weathering-and-aging of TNT in soil.
Note: Concentrations are based on ACN extraction (U.S. EPA Method 8330A) or water extraction (ATCLP).

The logistic Gompertz model had the best fit for the adult survival data. The EC_{20} and EC_{50} values for adult survival based on ACN-extractable concentrations were 179 and 362 mg kg^{-1}, respectively, for TNT FA in SSL, and 103 and 142 mg kg^{-1}, respectively, for TNT W-A in SSL soil. The EC_{20} and EC_{50} values for adult survival based on ATCLP-extractable concentrations were 132 and 275 mg kg^{-1}, respectively, for TNT FA in SSL, and 32 and 44 mg kg^{-1}, respectively, for TNT W-A in SSL soil (Table 21). Evaluation of these data showed that weathering-and-aging of TNT in SSL soil significantly (95% CI basis) increased acute toxicity for *E. crypticus* based on the EC_{50} values for adult survival.

Juvenile production was the more sensitive measurement endpoint for assessing TNT toxicity for *E. crypticus* in FA SSL compared with adult survival. Juvenile production was

stimulated in the lowest positive TNT concentration of 40 mg kg^{-1} (Figure 3), resulting in a 24% increase in the mean number of juveniles compared with the carrier control (Table 15). The increase was statistically significant (p = 0.003), producing an unbounded LOEC value of 40 mg kg^{-1} and bounded NOAEC and LOAEC values of 62 and 85 mg kg^{-1}, respectively, based on ACN-extractable TNT. The bounded NOAEC and LOAEC values for juvenile production based on ATCLP-extractable TNT in FA SSL were 40 and 59 mg kg^{-1}, respectively (Table 22). The bounded NOAEC and LOAEC values for TNT W-A in SSL soil were 3 and 46 mg kg^{-1}, respectively, based on ACN-extractable TNT and 0.4 and 14 mg kg^{-1}, respectively, based on ATCLP-extractable TNT (Table 22).

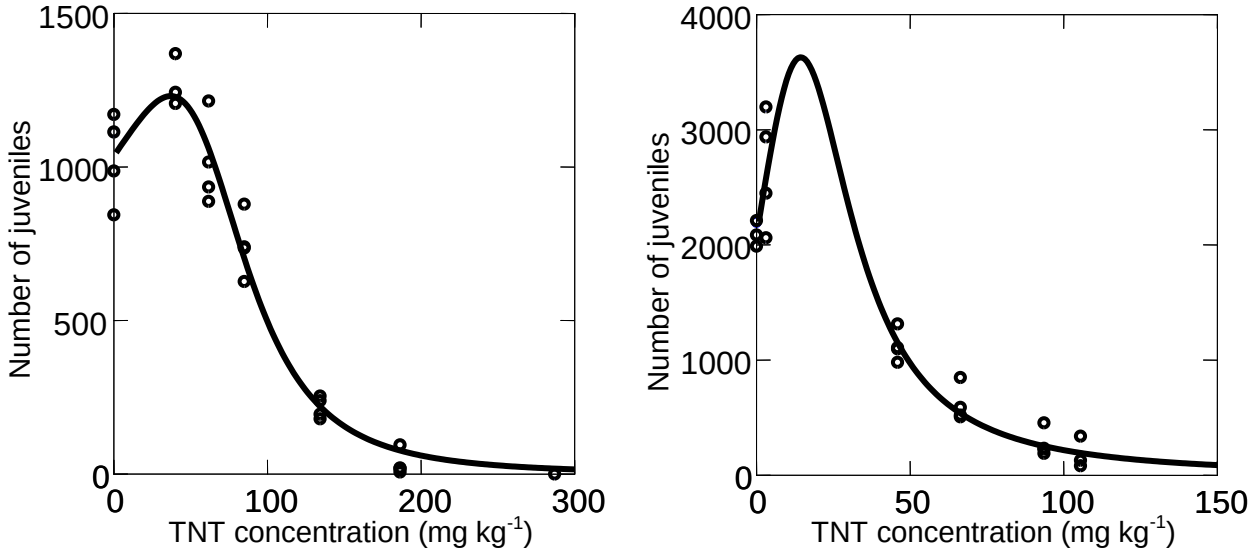

Figure 3. Effects of TNT FA (left) or W-A (right) in SSL soil on production of juveniles by *E. crypticus*. Modified from Kuperman et al. (2006e).

The logistic hormetic model had the best fit for reproduction data from toxicity tests with TNT FA in SSL soil, due to stimulation of juvenile production at the lower treatment concentrations (Figure 3). The EC_{20} and EC_{50} values for production of juveniles were 77 and 98 mg kg^{-1}, respectively, based on ACN-extractable TNT and 52 and 69 mg kg^{-1}, respectively, based on ATCLP-extractable TNT (Table 22). This model also had the best fit for reproduction data from toxicity tests with TNT W-A in SSL soil (Figure 3). The EC_{20} and EC_{50} values for production of juveniles were 38 and 48 mg kg^{-1}, respectively, based on ACN-extractable TNT and 12 and 15 mg kg^{-1}, respectively, based on ATCLP-extractable TNT (Table 22). Weathering-and-aging of TNT in SSL soil significantly increased reproductive toxicity for *E. crypticus* based on both the EC_{20} and EC_{50} values and their respective 95% CIs (Table 22).

Table 22. Chronic Ecotoxicological Benchmarks for TNT FA or W-A in SSL Soil Determined for Production of Juveniles by *E. crypticus*[*]

Ecotoxicological Parameter	TNT FA (mg kg^{-1})	TNT W-A (mg kg^{-1})
	ACN Extraction	
NOAEC	62	3
p	0.820	0.002
LOAEC	85	46
p	<0.0001	<0.0001
EC_{20}	77	38[†]
CI (95%)	70–84	31–44
EC_{50}	98	48[†]
CI (95%)	91–106	43–54
Model used	Hormetic	Hormetic
R^2	0.987	0.980
	ATCLP Extraction (Aqueous)	
NOAEC	40	0.4
p	0.820	0.002
LOAEC	59	14
p	<0.0001	<0.0001
EC_{20}	52	12[†]
CI (95%)	47–58	10–14
EC_{50}	69	15[†]
CI (95%)	63–75	13–16
Model used	Hormetic	Hormetic
R^2	0.985	0.979

[*]Modified from Kuperman et al. (2006e).
[†]Statistically significant (95% CI basis) increase in toxicity following weathering-and-aging of TNT in soil.
Note: Concentrations are based on ACN extraction (U.S. EPA Method 8330A) or water extraction (ATCLP).

R^2 values from nonlinear regression analyses of the reproduction toxicity data for TNT FA or TNT W-A in SSL soil, based on ACN- or ATCLP-extractable TNT concentrations, were compared to determine which chemical measure of exposure better correlated with toxicity. The values of coefficients were 0.987 and 0.985 for ACN and ATCLP extraction, respectively, in tests with TNT FA in SSL and 0.980 and 0.979 for ACN and ATCLP extraction, respectively, in tests with TNT W-A in SSL (Table 22). These comparisons showed that coefficients were very similar for both extraction types in either FA or W-A treatments, which indicates that data from both extraction methods had excellent correlation with the toxicity data for juvenile production, and that neither method had an advantage for characterizing bioavailability of TNT to *E. crypticus* in SSL soil.

3.3.3 TNT Toxicity in KCL Soil

Ecotoxicological responses of *E. crypticus* to TNT FA and W-A in KCL soil are shown in Table 16. Both adult survival and juvenile production were affected in TNT-amended KCL soil within the concentration ranges selected for definitive tests (Table 16). Acute ecotoxicological benchmarks for TNT, based on analytically determined concentration values, are summarized in Table 23. For adult survival, the bounded NOEC and LOEC values based on ACN-extractable concentrations were 300 and 380 mg kg^{-1}, respectively, for TNT FA in KCL and 147 and 313 mg kg^{-1}, respectively, for TNT W-A in KCL soil. Values based on ATCLP-extractable TNT concentrations were 194, 235, 71, and 172 mg kg^{-1}, respectively (Table 23).

Table 23. Acute Ecotoxicological Benchmarks for TNT FA or W-A in KCL Soil Determined for Survival of Adult *E. crypticus*

Ecotoxicological Parameter	TNT FA (mg kg^{-1})	TNT W-A (mg kg^{-1})
	ACN Extraction	
NOEC	300	147
p	0.647	1.0
LOEC	380	313
p	0.028	<0.0001
EC_{20}	393	331
CI (95%)	256–531	179–482
EC_{50}	714	836
CI (95%)	589–839	432–1240
Model used	Gompertz	Gompertz
R^2	0.976	0.989
	ATCLP Extraction (Aqueous)	
NOEC	194	71
p	0.647	1.0
LOEC	235	172
p	0.028	<0.0001
EC_{20}	257	174
CI (95%)	196–319	93–254
EC_{50}	373	458
CI (95%)	332–414	230–686
Model used	Gompertz	Gompertz
R^2	0.975	0.990

Note: Concentrations are based on ACN extraction (U.S. EPA Method 8330A) or water extraction (ATCLP).

The logistic Gompertz model had the best fit for the adult survival data. The acute EC_{20} and EC_{50} values based on ACN-extractable concentrations were 393 and 714 mg kg^{-1}, respectively, for TNT FA in KCL and 331 and 836 mg kg^{-1}, respectively, for TNT W-A in KCL soil. The EC_{20} and EC_{50} values for adult survival based on ATCLP-extractable concentrations were 257 and 373 mg kg^{-1}, respectively, for TNT FA in KCL and 174 and 458 mg kg^{-1}, respectively, for TNT W-A in KCL soil (Table 23). Weathering-and-aging of TNT in KCL soil did not significantly affect acute toxicity for *E. crypticus* based on 95% CIs for either the EC_{20} or EC_{50} values (Table 23).

Juvenile production was the more sensitive measurement endpoint for assessing TNT toxicity for *E. crypticus* in KCL compared with adult survival. The mean number of juveniles was significantly decreased ($p < 0.0001$) in the lowest positive TNT treatment in FA KCL compared with the acetone control, producing the unbounded LOEC values of 41 and 18 mg kg^{-1}, based on ACN- and ATCLP-extractable TNT concentrations, respectively (Table 24). The bounded NOEC and LOEC values for TNT W-A in KCL soil were 147 and 313 mg kg^{-1}, respectively, based on ACN-extractable TNT and 71 and 172 mg kg^{-1}, respectively, based on ATCLP-extractable TNT (Table 24).

Table 24. Chronic Ecotoxicological Benchmarks for TNT FA or W-A in KCL Soil Determined for Production of Juveniles by *E. crypticus*

Ecotoxicological Parameter	TNT FA (mg kg^{-1})	TNT W-A (mg kg^{-1})
	ACN Extraction	
NOEC	<41	147
p	ND	0.052
LOEC	41[†]	313
p	<0.0001	<0.0001
EC_{20}	133	157
CI (95%)	108–159	121–193
EC_{50}	197	189
CI (95%)	179–215	153–225
Model used	Gompertz	Hormetic
R^2	0.970	0.980
	ATCLP Extraction (Aqueous)	
NOEC	<18	71
p	ND	0.052
LOEC	18[†]	172
p	<0.0001	<0.0001
EC_{20}	72	75
CI (95%)	55–90	68–82
EC_{50}	116	93
CI (95%)	102–129	69–117
Model used	Gompertz	Hormetic
R^2	0.964	0.979

[†]Unbounded LOEC.

Note: Concentrations are based on ACN extraction (U.S. EPA Method 8330A) or water extraction (ATCLP).

ND, could not be determined within concentration range tested.

The logistic Gompertz model had the best fit for the juvenile production data from toxicity tests with TNT FA in KCL soil (Figure 4). The EC_{20} and EC_{50} values for production of juveniles were 133 and 197 mg kg^{-1}, respectively, based on ACN-extractable TNT and 72 and 116 mg kg^{-1}, respectively, based on ATCLP-extractable TNT (Table 24). The logistic hormetic model had the best fit for the data from toxicity tests with TNT W-A in KCL soil, due to a nonsignificant ($p \geq 0.139$) stimulation of juvenile production at the two lowest TNT concentrations (Figure 4). The EC_{20} and EC_{50} values for production of juveniles in this test were 157 and 189 mg kg^{-1}, respectively, based on ACN-extractable TNT and 75 and 93 mg kg^{-1}, respectively, based on ATCLP-extractable TNT (Table 24). Weathering-and-aging of TNT in KCL soil did not significantly affect reproductive toxicity for *E. crypticus* based on 95% CIs for either the EC_{20} or EC_{50} values (Table 24).

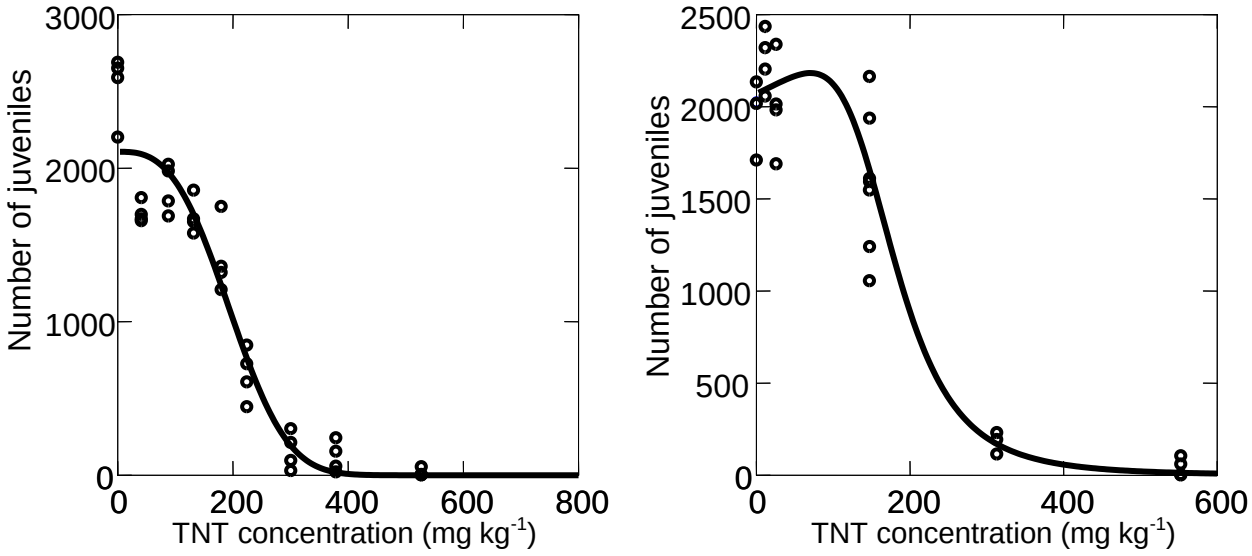

Figure 4. Effects of TNT FA (left) or W-A (right) in KCL soil on production of juveniles by *E. crypticus*.

R^2 values from nonlinear regression analyses of the reproduction toxicity data for TNT FA or TNT W-A in KCL soil, based on ACN- or ATCLP-extractable TNT concentrations, were compared to determine which chemical measure of exposure better correlated with toxicity. The values of coefficients were 0.970 and 0.964 for ACN and ATCLP extraction, respectively, in tests with TNT FA in KCL and 0.980 and 0.979 for ACN and ATCLP extraction, respectively, in tests with TNT W-A in KCL (Table 24). These comparisons showed that coefficients were very similar for both extraction types in either FA or W-A treatments, indicating that data from both extraction methods had excellent correlation with the toxicity data for juvenile production, and that neither method had an advantage for characterizing bioavailability of TNT to *E. crypticus* in KCL soil.

3.3.4 TNT Toxicity in RCL Soil

Ecotoxicological responses of *E. crypticus* to TNT FA and W-A in RCL soil are shown in Table 17. Both adult survival and juvenile production were affected in TNT-amended RCL soil within the concentration ranges selected for definitive tests (Table 17). Acute ecotoxicological benchmarks for TNT, based on analytically determined concentration values, are summarized in Table 25. For adult survival, the bounded NOEC and LOEC values based on ACN-extractable concentrations were 267 and 330 mg kg^{-1}, respectively, for TNT FA in RCL and 142 and 253 mg kg^{-1}, respectively, for TNT W-A in RCL soil. Values based on ATCLP-extractable TNT concentrations were 152, 194, 72, and 135 mg kg^{-1}, respectively (Table 25).

The logistic Gompertz model had the best fit for the adult survival data. The EC_{20} and EC_{50} values for adult survival based on ACN-extractable concentrations were 371 and 1246 mg kg^{-1}, respectively, for TNT FA in RCL and 214 and 263 mg kg^{-1}, respectively, for TNT W-A in KCL soil. The EC_{20} and EC_{50} values for adult survival based on ATCLP-extractable

concentrations were 195 and 382 mg kg^{-1}, respectively, for TNT FA in RCL and 111 and 140 mg kg^{-1}, respectively, for TNT W-A in RCL soil (Table 25). Weathering-and-aging of TNT in RCL soil significantly increased acute toxicity for *E. crypticus* based on the EC_{50} values and respective 95% CIs (Table 25).

Table 25. Acute Ecotoxicological Benchmarks for TNT FA or W-A in RCL Soil Determined for Survival of Adult *E. crypticus*

Ecotoxicological Parameter	TNT FA (mg kg^{-1})	TNT W-A (mg kg^{-1})
	ACN Extraction	
NOEC	267	142
p	0.128	0.539
LOEC	330	253
p	0.001	<0.0001
EC_{20}	371	214
CI (95%)	154–588	184–244
EC_{50}	1246	263*
CI (95%)	737–1755	251–274
Model used	Gompertz	Gompertz
R^2	0.989	0.997
	ATCLP Extraction (Aqueous)	
NOEC	152	72
p	0.128	0.539
LOEC	194	135
p	0.001	<0.0001
EC_{20}	195	111*
CI (95%)	139–251	93–130
EC_{50}	382	140*
CI (95%)	290–474	133–147
Model used	Gompertz	Gompertz
R^2	0.991	0.997

*Statistically significant (95% CI basis) increase in toxicity following weathering-and-aging of TNT in soil.
Note: Concentrations are based on ACN extraction (U.S. EPA Method 8330A) or water extraction (ATCLP).

Juvenile production was the more sensitive measurement endpoint for assessing TNT toxicity for *E. crypticus* in RCL compared with adult survival. The bounded NOEC and LOEC values for TNT FA in RCL soil were 168 and 267 mg kg^{-1}, respectively, based on ACN-extractable TNT and 85 and 152 mg kg^{-1}, respectively, based on ATCLP-extractable TNT (Table 26). Juvenile production was stimulated in the two lowest positive concentrations of TNT W-A in RCL soil, which resulted in the respective 3 and 17% increases in the mean number of

juveniles compared with carrier control (Figure 5). The increase in 93 mg kg^{-1} treatment was statistically significant (p = 0.022), producing the NOEC and LOEC values of 35 and 93 mg kg^{-1}, respectively and the NOAEC and LOAEC values of 142 and 253 mg kg^{-1}, respectively, based on ACN-extractable TNT. The NOAEC and LOAEC values for juvenile production based on ATCLP-extractable TNT W-A in RCL soil were 72 and 135 mg kg^{-1}, respectively (Table 26).

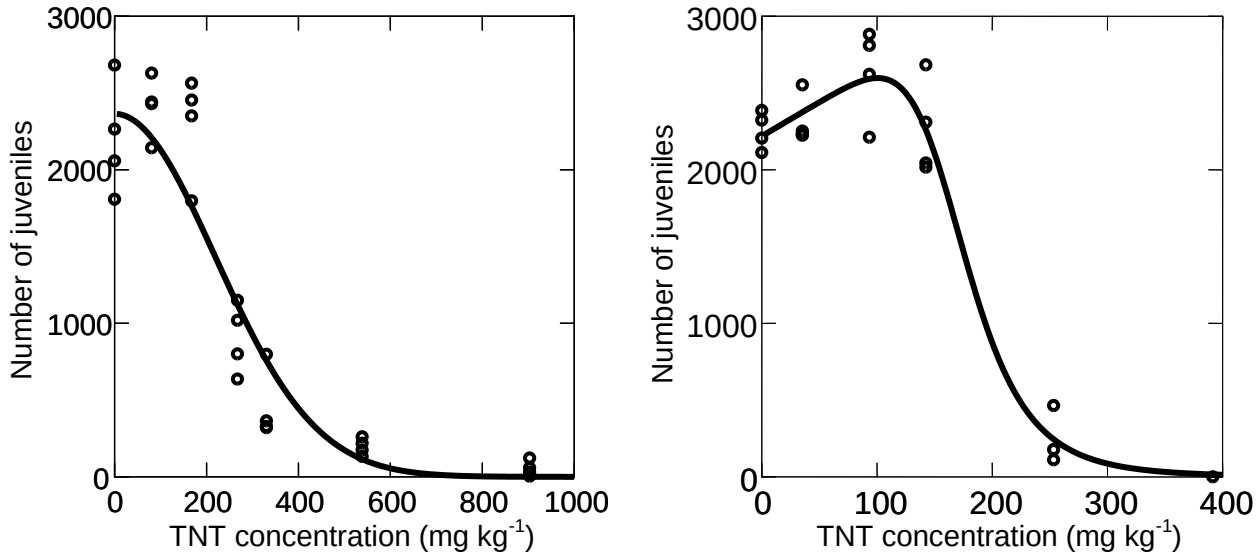

Figure 5. Effects of TNT FA (left) or W-A (right) in RCL soil on production of juveniles by *E. crypticus*.

The logistic Gompertz model had the best fit for the juvenile production data from toxicity tests with TNT FA in RCL soil (Figure 5). The EC_{20} and EC_{50} values for production of juveniles were 198 and 258 mg kg^{-1}, respectively, based on ACN-extractable TNT and 105 and 145 mg kg^{-1}, respectively, based on ATCLP-extractable TNT (Table 26). The logistic hormetic model had the best fit for the data from toxicity tests with TNT W-A in RCL soil, due to stimulation of juvenile production at the two lowest concentrations of TNT (Figure 5). The EC_{20} and EC_{50} values for production of juveniles in this test were 128 and 189 mg kg^{-1}, respectively, based on ACN-extractable TNT and 64 and 97 mg kg^{-1}, respectively, based on ATCLP-extractable TNT (Table 26). Weathering-and-aging of TNT in RCL soil significantly increased reproductive toxicity for *E. crypticus* based on both the EC_{20} and EC_{50} values and their respective 95% CIs (Table 26).

Table 26. Chronic Ecotoxicological Benchmarks for TNT FA or W-A in RCL Soil Determined for Production of Juveniles by *E. crypticus*

Ecotoxicological Parameter	TNT FA (mg kg^{-1})	TNT W-A (mg kg^{-1})
	ACN Extraction	
NOEC	168	142
p	0.608	0.964
LOEC	267	253
p	<0.0001	<0.0001
EC_{20}	198	128*
CI (95%)	163–234	95–161
EC_{50}	258	189*
CI (95%)	237–279	172–207
Model used	Gompertz	Hormetic
R^2	0.974	0.991
	ATCLP Extraction (Aqueous)	
NOEC	85	72
p	0.608	0.964
LOEC	152	135
p	<0.0001	<0.0001
EC_{20}	105	64*
CI (95%)	81.9–128	46.5–81.7
EC_{50}	145	97*
CI (95%)	131–159	88–107
Model used	Gompertz	Hormetic
R^2	0.975	0.992

*Statistically significant (95% CI basis) increase in toxicity following weathering-and-aging of TNT in soil.
Note: Concentrations of TNT are based on ACN extraction (U.S. EPA Method 8330A) or water extraction (ATCLP).

R^2 values from nonlinear regression analyses of the reproduction toxicity data for TNT FA or W-A in KCL soil, based on ACN- or ATCLP-extractable TNT concentrations, were compared to determine which chemical measure of exposure better correlated with toxicity. The values of coefficients were 0.974 and 0.975 for ACN and ATCLP extraction, respectively, in tests with TNT FA in RCL and 0.991 and 0.992 for ACN and ATCLP extraction, respectively, in tests with TNT W-A in KCL (Table 26). These comparisons showed that coefficients were very similar for both extraction types in either FA or W-A treatments, indicating that data from both extraction methods had excellent correlation with the toxicity data for juvenile production, and that neither method had an advantage for characterizing bioavailability of TNT to *E. crypticus* in KCL soil.

3.3.5 TNT Toxicity in WCL Soil

Ecotoxicological responses of *E. crypticus* to TNT FA and to W-A in WCL soil are shown in Table 18. Both adult survival and juvenile production were affected in TNT-amended RCL soil within the concentration ranges selected for definitive tests (Table 18). Acute ecotoxicological benchmarks for TNT, based on analytically determined concentration values, are summarized in Table 27. For adult survival, the bounded NOEC and LOEC values based on ACN-extractable concentrations were 387 and 433 mg kg^{-1}, respectively, for TNT FA in WCL and 528 and 695 mg kg^{-1}, respectively, for TNT W-A in WCL soil. Values based on ATCLP-extractable TNT concentrations were 159, 178, 58, and 179 mg kg^{-1}, respectively (Table 27).

Table 27. Acute Ecotoxicological Benchmarks for TNT FA or W-A in WCL Soil Determined for Survival of Adult *E. crypticus*

Ecotoxicological Parameter	TNT FA (mg kg^{-1})	TNT W-A (mg kg^{-1})
	ACN Extraction	
NOEC	387	528
p	1.0	0.422
LOEC	433	695
p	0.016	<0.0001
EC_{20}	485	670
CI (95%)	180–790	650–691
EC_{50}	1087	796
CI (95%)	683–1490	739–854
Model used	Gompertz	Gompertz
R^2	0.961	0.999
	ATCLP Extraction (Aqueous)	
NOEC	159	58
p	1.0	1.0
LOEC	178	179
p	0.016	0.031
EC_{20}	224	257
CI (95%)	73–376	245–270
EC_{50}	568	335
CI (95%)	331–805	298–372
Model used	Gompertz	Gompertz
R^2	0.963	0.999

Note: Concentrations are based on ACN extraction (U.S. EPA Method 8330A) or water extraction (ATCLP).

The logistic Gompertz model had the best fit for the adult survival data. The EC_{20} and EC_{50} values for adult survival based on ACN-extractable concentrations were 485 and 1087 mg kg^{-1}, respectively, for TNT FA in WCL and 670 and 796 mg kg^{-1}, respectively, for TNT W-A in WCL soil. The EC_{20} and EC_{50} values for adult survival based on ATCLP-extractable concentrations were 224 and 568 mg kg^{-1}, respectively, for TNT FA in WCL and 257 and 335 mg kg^{-1}, respectively, for TNT W-A in WCL soil (Table 27). Weathering-and-aging of TNT in WCL soil did not significantly affect acute toxicity for *E. crypticus* based on 95% CI for either the EC_{20} or EC_{50} values (Table 27).

Juvenile production was the more sensitive measurement endpoint for assessing TNT toxicity for *E. crypticus* in WCL compared with adult survival. The bounded NOEC and LOEC values for TNT FA in WCL soil were 86 and 245 mg kg^{-1}, respectively, based on ACN-extractable TNT and 11 and 83 mg kg^{-1}, respectively, based on ATCLP-extractable TNT (Table 28). The bounded NOEC and LOEC values for TNT W-A in WCL soil were 30 and 165 mg kg^{-1}, respectively, based on ACN-extractable TNT and 5 and 45 mg kg^{-1}, respectively, based on ATCLP-extractable TNT (Table 28).

The logistic Gompertz model had the best fit for the juvenile production data from toxicity tests with TNT either FA or W-A in WCL soil (Figure 6). The EC_{20} and EC_{50} values for production of juveniles were 343 and 514 mg kg^{-1}, respectively, based on ACN-extractable TNT and 136 and 230 mg kg^{-1}, respectively, based on ATCLP-extractable TNT FA in WCL soil (Table 28). The EC_{20} and EC_{50} values for TNT W-A in WCL soil were 123 and 289 mg kg^{-1}, respectively, based on ACN-extractable TNT and 35 and 93 mg kg^{-1}, respectively, based on ATCLP-extractable TNT (Table 28). Weathering-and-aging of TNT in WCL soil significantly increased reproductive toxicity for *E. crypticus* based on both the EC_{20} and EC_{50} values and their respective 95% CIs (Table 28).

R^2 values from nonlinear regression analyses of the reproduction toxicity data for TNT FA or W-A in WCL soil, based on ACN- or ATCLP-extractable TNT concentrations, were compared to determine which chemical measure of exposure better correlated with toxicity. The values of coefficients were 0.970 and 0.967 for ACN and ATCLP extraction, respectively, in tests with TNT FA in WCL and 0.974 and 0.977 for ACN and ATCLP extraction, respectively, in tests with TNT W-A in WCL (Table 28). These comparisons showed that coefficients were very similar for both extraction types in either FA or W-A treatments, indicating that data from both extraction methods had excellent correlation with the toxicity data for juvenile production, and that neither method had an advantage for characterizing bioavailability of TNT to *E. crypticus* in WCL soil.

Table 28. Chronic Ecotoxicological Benchmarks for TNT FA or W-A in WCL Soil Determined for Production of Juveniles by *E. crypticus*

Ecotoxicological Parameter	TNT FA (mg kg^{-1})	TNT W-A (mg kg^{-1})
	ACN Extraction	
NOEC	86	30
p	0.140	0.649
LOEC	245	165
p	0.049	0.008
EC_{20}	343	123*
CI (95%)	277–408	67–180
EC_{50}	514	289*
CI (95%)	464–564	220–358
Model used	Gompertz	Gompertz
R^2	0.970	0.974
	ATCLP Extraction (Aqueous)	
NOEC	11	5
p	0.140	0.719
LOEC	83	45
p	0.002	0.005
EC_{20}	136	35*
CI (95%)	99–172	18–52
EC_{50}	230	93*
CI (95%)	199–262	69–117
Model used	Gompertz	Gompertz
R^2	0.967	0.977

*Statistically significant (95% CI basis) increase in toxicity following weathering-and-aging of TNT in soil.

Note: Concentrations of TNT are based on ACN extraction (U.S. EPA Method 8330A) or water extraction (ATCLP);

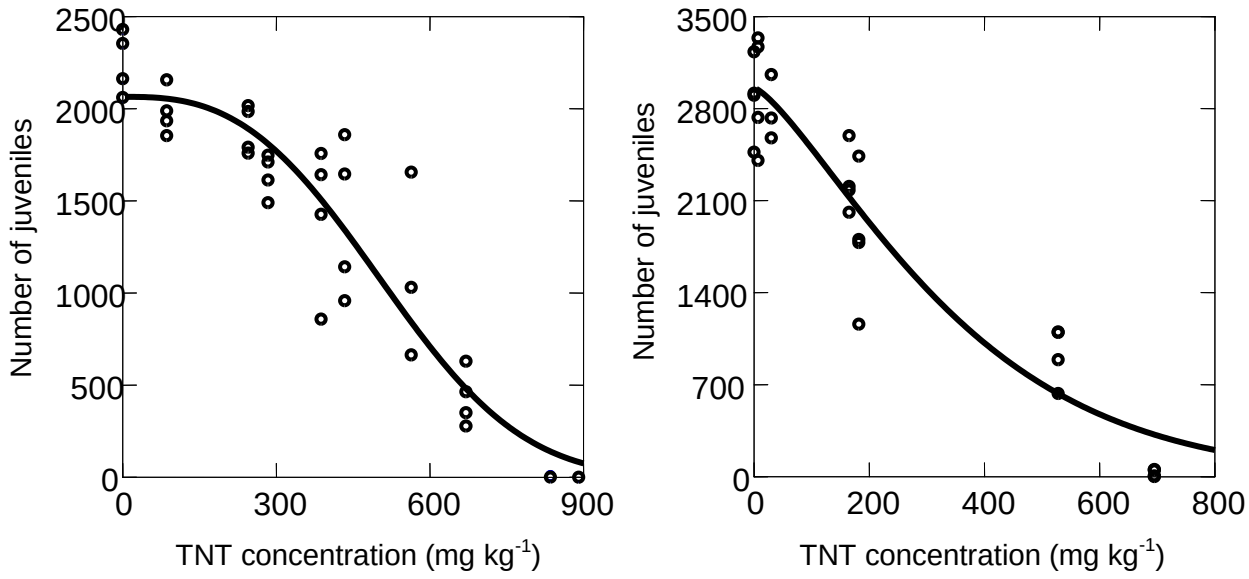

Figure 6. Effects of TNT FA (left) or W-A (right) in WCL soil on production of juveniles by *E. crypticus*.

3.4 Effects of RDX on the Potworm *E. crypticus*

Test results complied with the validity criteria for negative controls defined in the ISO 16387 (2004) test guideline (Tables 29–33). Mean adult survival ranged from 98 to 100% in RDX studies with FA soil and from 95 to 100% in studies with RDX W-A in soil. The mean number of juveniles ranged from 556 to 1624 in studies with FA soil and from 293 to 2802 in studies with RDX W-A in soil. All CV values for the numbers of juveniles produced by *E. crypticus* in negative controls were <50%, as required by the ISO 16387 test guideline, and ranged from 3 to 24% in studies with RDX FA in soil and from 10 to 29% in studies with RDX W-A in soil. The mean numbers of juveniles in positive controls of studies with FA RDX ranged from 33 to 42% of respective SSL controls and from 39 to 45% of respective SSL controls in studies with RDX W-A in soil (negative control data were used in studies with SSL soil). These positive control data were consistent with the baseline established for the laboratory culture of *E. crypticus*. Direct comparisons of the results of positive control treatments are not possible because ISO 16387 was a relatively new method at the time these tests were conducted, and also because ISO 16387 was originally optimized for testing chemicals with a different potworm species, *E. albidus*, in the OECD artificial soil. In addition, reference values for *E. crypticus* exposures in natural soils were not available from the literature. This issue was resolved later by a proposal to use boric acid as a universal reference toxicant and by establishing warning charts to continuously monitor the health and performance of test species maintained in ECBC laboratory cultures (Amorim et al., 2009; Kuperman et al., 2006a, 2008, 2009c). Compliance with the test validity criteria confirmed that the toxicological effects determined in the definitive tests were attributable to the RDX treatments.

Table 29. Adult Survival and Juvenile Production by *E. crypticus* Exposed to RDX FA or W-A in TSL Soil

RDX FA Treatment (mg kg^{-1})	Mean		SE	RDX W-A Treatment (mg kg^{-1})	Mean		SE
	Adults	Juveniles			Adults	Juveniles	
Negative control	10	556	66	Negative control	9.8	293	43
Acetone control	10	951	88	Acetone control	10	1,209	79
Positive control	9.3	831	44	Positive control	9.5	649	30
SSL soil control	10	1,973	144	SSL soil control	10	1,655	123
1,200	10	766	36	1,060	9.8	1,140	40
2,200	10	701	74	2,300	9.5	1,252	163
4,500	9.8	641	44	5,560	10	1,836	243
6,300	8.3	256	75	7,000	9.8	1,421	125
10,000	0.0	0	0	15,000	9.5	735	78

Notes: RDX concentrations are based on ACN extraction using U.S. EPA Method 8330A. Toxicity data are means (n = 4).

Table 30. Adult Survival and Juvenile Production by *E. crypticus* Exposed to RDX FA or W-A in SSL Soil*

RDX FA Treatment (mg kg^{-1})	Mean		SE	RDX W-A Treatment (mg kg^{-1})	Mean		SE
	Adults	Juveniles			Adults	Juveniles	
Negative control	10	1,278	35	Negative control	10	1,120	84
Acetone control	10	1,314	80	Acetone control	10	1,748	30
Positive control	10	420	24	Positive control	10	489	33
300	9.8	1,395	34	NT	NT	NT	NT
660	10	1,336	50	NT	NT	NT	NT
1,200	10	1,170	13	1050	10	1,550	55
2,200	10	1,032	58	2,380	9.8	1,587	87
4,560	10	1,009	17	3,985	10	1,434	65
10,000	10	974	59	9,550	10	1,434	53
21,400	10	909	53	18,350	9.8	1,264	13

*Data were reported initially in Kuperman et al. (2004a) and are included in this report for convenience of comparison with other soils.

Notes: Toxicity data are means (n = 4). RDX concentrations are based on ACN extraction using U.S. EPA Method 8330A.

NT, not tested in this study.

Table 31. Adult Survival and Juvenile Production by *E. crypticus* Exposed to RDX FA or W-A in KCL Soil

RDX FA Treatment (mg kg^{-1})	Mean		SE	RDX W-A Treatment (mg kg^{-1})	Mean		SE
	Adults	Juveniles			Adults	Juveniles	
Negative control	9.8	1525	54	Negative control	10	1740	86
Acetone control	9.9	1566	39	Acetone control	9.8	1264	81
Positive control	10	425	103	Positive control	9.3	645	68
SSL soil control	9.5	1113	53	SSL soil control	9.0	1439	60
12,300	10	1550	60	9840	9.6	2133	142

Notes: RDX concentrations are based on ACN extraction using U.S. EPA Method 8330A. Toxicity data are means (n = 4 in negative, positive, and SSL soil controls; n = 8 in the remaining treatments).

Table 32. Adult Survival and Juvenile Production by *E. crypticus* Exposed to RDX FA or W-A in RCL Soil

RDX FA Treatment (mg kg^{-1})	Mean		SE	RDX W-A Treatment (mg kg^{-1})	Mean		SE
	Adults	Juveniles			Adults	Juveniles	
Negative control	10	801	41	Negative control	10	1718	232
Acetone control	10	1333	49	Acetone control	9.6	2041	65
Positive control	10	425	103	Positive control	9.3	645	68
SSL soil control	9.5	1113	53	SSL soil control	9.0	1439	60
10,600	9.8	1453	101	9720	9.3	1830	80

Notes: RDX concentrations are based on ACN extraction using U.S. EPA Method 8330A. Toxicity data are means (n = 4 in negative, positive, and SSL soil controls; n = 8 in the remaining treatments).

Table 33. Adult Survival and Juvenile Production by *E. crypticus* Exposed to RDX FA or W-A in WCL Soil

RDX FA Treatment (mg kg^{-1})	Mean		SE	RDX W-A Treatment (mg kg^{-1})	Mean		SE
	Adults	Juveniles			Adults	Juveniles	
Negative control	10	1624	25	Negative control	9.5	2802	140
Acetone control	9.9	1542	29	Acetone control	8.6	3276	171
Positive control	10	425	103	Positive control	9.3	645	68
SSL soil control	9.5	1113	53	SSL soil control	9.0	1439	60
16,240	9.5	1425	40	10,700	9.5	3009	82

Notes: RDX concentrations are based on ACN extraction using U.S. EPA Method 8330A. Toxicity data are means (n = 4 in negative, positive, and SSL soil controls; n = 8 in the remaining treatments).

3.4.1 Effects of RDX in TSL Soil

Ecotoxicological responses of *E. crypticus* to RDX FA and W-A in TSL soil are shown in Table 29. Ecotoxicological benchmarks for RDX, based on analytically determined concentration values, are summarized in Table 34. Survival of adult *E. crypticus* was affected within the concentration range selected for definitive test with RDX FA in TSL soil, producing the bounded NOEC and LOEC values of 4500 and 6300 mg kg^{-1}, respectively. The logistic Gompertz model had the best fit for the adult survival data, producing the EC_{20} and EC_{50} values of 6414 and 7511 mg kg^{-1}, respectively. Exposure of *E. crypticus* to RDX W-A in TSL soil up to and including the greatest concentration tested in this study did not affect adult survival and produced an unbounded NOEC of 15,000 mg kg^{-1}.

Table 34. Ecotoxicological Benchmarks for RDX FA and W-A in TSL Soil Determined in Definitive Tests with *E. crypticus*

Ecotoxicological Parameter	RDX FA (mg kg^{-1})	RDX W-A (mg kg^{-1})
	Adult Survival	
NOEC	4500	15,000‡
p	0.632	0.150
LOEC	6300	>15,000
p	0.003	ND
EC_{20}	6414	>15,236
CI (95%)	5980-6848	ND
EC_{50}	7511	>15,236
CI (95%)	6250-8772	ND
Model used	Gompertz	ND
R^2	0.995	ND
	Juvenile Production	
NOEC	<1200	1060
p	ND	0.616
LOEC	1200‡‡	15,000†
p	0.045	0.003
EC_{20}	4300	13,000*
CI (95%)	3210-5400	10,000-16,300
EC_{50}	5610	17,000*
CI (95%)	5000-6000	12,000-22,000
Model used	Gompertz	Hormetic
R^2	0.961	0.969

*Statistically significant (95% CI basis) decrease in toxicity following weathering-and-aging of RDX in soil.
Note: Concentrations are based on ACN extraction (U.S. EPA Method 8330A).
ND, could not be determined within the concentration range tested.

Production of juveniles by *E. crypticus* was affected within the concentration ranges selected for definitive tests with RDX FA and W-A in TSL soil (Table 29). Juvenile production was the more sensitive measurement endpoint for assessing RDX effects on *E. crypticus* in TSL compared with adult survival. Exposure of *E. crypticus* to the first positive RDX concentration in FA TSL soil resulted in a 19% decrease in the mean number of juveniles compared with the acetone control. The decrease was statistically significant ($p = 0.045$), producing an unbounded LOEC of 1200 mg kg^{-1}. In definitive tests with RDX W-A in TSL soil, production of juveniles by *E. crypticus* was unaffected at 1060 mg kg^{-1} (bounded NOEC; $p = 0.616$) and stimulated in treatments up to and including 7000 mg kg^{-1} (Figure 6), producing the bounded LOEC ($p = 0.001$) and LOAEC ($p = 0.003$) values of 5560 and 15,000 mg kg^{-1}, respectively for RDX W-A in TSL soil (Table 34).

The logistic Gompertz model had the best fit for the juvenile production data from definitive toxicity tests with RDX FA in TSL soil (Figure 7). The EC_{20} and EC_{50} values for production of juveniles were 4300 and 5610 mg kg^{-1}, respectively. The logistic hormetic model had the best fit for reproduction data from toxicity tests with RDX W-A in TSL soil, due to stimulation of juvenile production at the lower treatment concentrations described (Figure 6). The EC_{20} and EC_{50} values for production of juveniles were 13,000 and 17,000 mg kg^{-1}, respectively (Table 34). Weathering-and-aging of RDX in TSL soil significantly decreased reproductive toxicity for *E. crypticus* based on both the EC_{20} and EC_{50} values and their respective 95% CIs (Table 34).

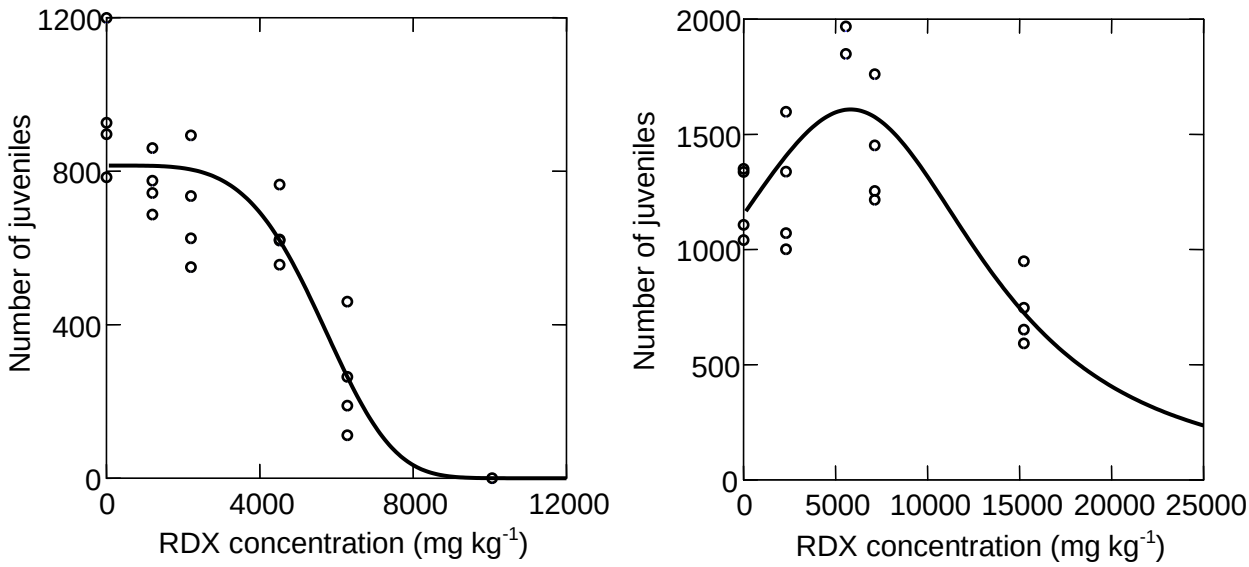

Figure 7. Effects of RDX FA (left) or W-A (right) in TSL soil on production of juveniles by *E. crypticus*.

3.4.2 Effects of RDX in SSL Soil

Ecotoxicological responses of *E. crypticus* to RDX FA in SSL soil and to RDX W-A in SSL soil are shown in Table 30. Ecotoxicological benchmarks for RDX, based on analytically determined concentration values, are summarized in Table 35. Survival of adult *E. crypticus* was not affected within the concentration ranges selected for definitive tests with RDX FA or W-A in SSL soil, producing the unbounded NOEC values of 21,400 and 18,350 mg kg^{-1}, respectively.

Production of juveniles by *E. crypticus* was affected within the concentration ranges selected for definitive tests with RDX FA in SSL soil and for definitive tests with RDX W-A in SSL soil (Table 30). Juvenile production was the more sensitive measurement endpoint for assessing RDX effects on *E. crypticus* in SSL compared with adult survival. The bounded NOEC and LOEC values for RDX FA in SSL soil were 1200 and 2200 mg kg^{-1}, respectively (Table 35). Production of juveniles by *E. crypticus* was decreased by 11% in the first positive RDX concentration treatment, compared with acetone control in definitive tests with RDX W-A in SSL soil. The decrease was statistically significant ($p = 0.022$), producing an unbounded LOEC of 1050 mg kg^{-1}.

Table 35. Ecotoxicological Benchmarks for RDX FA and W-A in SSL Soil Determined in Definitive Tests with *E. crypticus*[*]

Ecotoxicological Parameter	RDX FA (mg kg^{-1})	RDX W-A (mg kg^{-1})
	Adult Survival	
NOEC	21,400[‡]	18,350[†]
LOEC	>21,400	>18,350
	Juvenile Production	
NOEC	1200	<1050[‡]
p	0.055	ND
LOEC	2200	1050
p	0.001	0.022
EC_{20}	3715	8800
CI (95%)	0–8100	761–16,834
EC_{50}	51,410	142,360
CI (95%)	6336–96,491	ND
Model used	Gompertz	Gompertz
R^2	0.990	0.995

[*]Modified from Kuperman et al. (2004a); included in this report for convenience of comparison with other soils.
[†]Unbounded NOEC.
[‡]Unbounded LOEC.
Note: RDX concentrations are based on ACN extraction from soil (U.S. EPA Method 8330A).
ND, could not be determined within the concentration range tested.

The logistic Gompertz model had the best fit for the juvenile production data from definitive toxicity test with RDX FA or W-A in SSL soil (Figure 8). The EC_{20} and EC_{50} values for production of juveniles were 3715 and 51,410 mg kg^{-1}, respectively, for RDX FA in SSL soil and 8800 and 142,360 mg kg^{-1}, respectively, for RDX W-A in SSL soil (Table 35). The greatest RDX concentrations used in the respective definitive tests resulted only in 31 and 28% reductions in the mean number of juveniles produced, compared to respective acetone control data. These levels of effect were sufficient to estimate the EC_{20} values that are acceptable for use in derivation of an Eco-SSL. However, the EC_{50} benchmarks estimated by nonlinear regression analyses were outside the range of RDX concentrations used in each definitive test, and each of these benchmarks estimated by extrapolation of data had wide 95% CIs (Table 35), indicating high uncertainty in these point estimates.

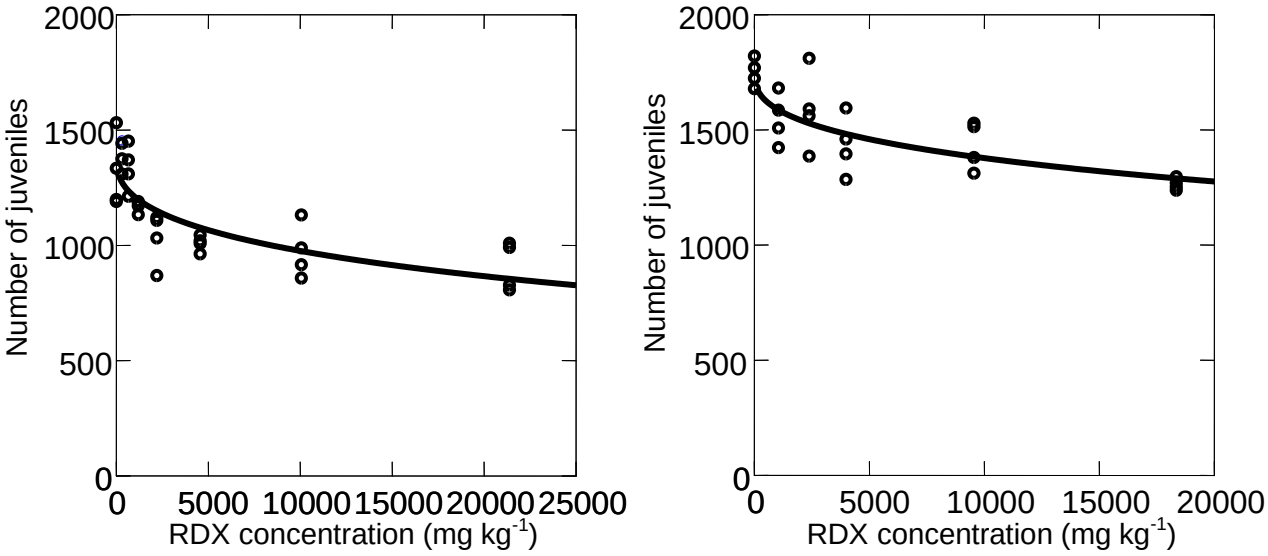

Figure 8. Effects of RDX FA (left) or W-A (right) in SSL soil on production of juveniles by *E. crypticus*. Data were reported initially in Kuperman et al. (2004a) and are included in this report for convenience of comparison with other soils.

3.4.3 Effects of RDX in KCL Soil

Ecotoxicological responses of *E. crypticus* to RDX FA and W-A in KCL soil are shown in Table 31. Ecotoxicological benchmarks for RDX, based on analytically determined concentration values, are summarized in Table 36. Neither survival of adults nor production of juveniles by *E. crypticus* was affected in the single greatest treatment concentration of RDX (compared with acetone control) used in the definitive limit tests with KCL soil. These results established unbounded NOEC values for either endpoint of 12,300 mg kg^{-1} for RDX FA in KCL soil and 9840 mg kg^{-1} for RDX W-A in KCL soil (Table 36).

Table 36. Ecotoxicological Benchmarks for RDX FA and W-A in KCL Soil Determined in Definitive Tests with *E. crypticus*

Ecotoxicological Parameter	RDX FA (mg kg^{-1})	RDX W-A (mg kg^{-1})
	Adult Survival	
NOEC	12,300*	9,840*
p	0.351	0.694
LOEC	>12,300	>9,840
	Juvenile Production	
NOEC	12,300*	9,840*
p	0.826	0.853
LOEC	>12,300	>9,840

*Unbounded NOEC.
Note: RDX concentrations are based on ACN extraction (U.S. EPA Method 8330A).

3.4.4 Effects of RDX in RCL Soil

Ecotoxicological responses of *E. crypticus* to RDX FA and W-A in RCL soil are shown in Table 32. Ecotoxicological benchmarks for RDX, based on analytically determined concentration values, are summarized in Table 37.

Table 37. Ecotoxicological Benchmarks for RDX FA and W-A in RCL Soil Determined in Definitive Tests with *E. crypticus*

Ecotoxicological Parameter	RDX FA (mg kg^{-1})	RDX W-A (mg kg^{-1})
	Adult Survival	
NOEC	10,600*	9,720*
p	0.170	0.248
LOEC	>10,600	>9,720
	Juvenile Production	
NOEC	10,600*	9,720*
p	0.304	0.059
LOEC	>10,600	>9,720

*Unbounded NOEC.
Note: RDX concentrations are based on ACN extraction (U.S. EPA Method 8330A).

Neither survival of adults nor production of juveniles by *E. crypticus* was affected in the single greatest treatment concentration of RDX (compared with acetone control) used in the definitive limit tests with RCL soil. These results established unbounded NOEC values for either endpoint of 10,600 mg kg^{-1} for RDX FA in RCL soil and 9720 mg kg^{-1} for RDX W-A in RCL soil (Table 37).

3.4.5 Effects of RDX in WCL Soil

Ecotoxicological responses of *E. crypticus* to RDX FA and W-A in WCL soil are shown in Table 33. Ecotoxicological benchmarks for RDX, based on analytically determined concentration values, are summarized in Table 38. Survival of adult *E. crypticus* was not affected in the single greatest treatment concentration of RDX (compared with acetone control) used in the definitive limit tests with WCL soil, producing unbounded NOEC values for this endpoint of 16,240 mg kg^{-1} for RDX FA in WCL soil and 10,700 mg kg^{-1} for RDX W-A in WCL soil (Table 38). Exposure of *E. crypticus* to RDX in FA WCL soil decreased production of juveniles by 7.7% compared with the acetone control. The decrease was statistically significant ($p = 0.031$) to establish an unbounded LOEC of 16,240 mg kg^{-1} (Table 38) but not sufficient to estimate the EC_{20} value. Exposure of *E. crypticus* to RDX W-A in WCL soil did not affect production of juveniles, establishing an unbounded NOEC of 16,240 mg kg^{-1}.

Table 38. Ecotoxicological Benchmarks for RDX FA and W-A in WCL Soil Determined in Definitive Tests with *E. crypticus*

Ecotoxicological Parameter	RDX FA (mg kg^{-1})	RDX W-A (mg kg^{-1})
	Adult Survival	
NOEC	16,240*	10,700*
p	0.411	0.112
LOEC	>16,240	>10,700
	Juvenile Production	
NOEC	<16,240	10,700*
p	ND	0.180
LOEC	16,240†	>10,700
p	0.031	ND

*Unbounded NOEC.
†Unbounded LOEC (7.7% decrease from acetone control).
Note: RDX concentrations are based on ACN extraction (U.S. EPA Method 8330A).
ND, could not be determined within the concentration range tested.

3.5 Effects of Soil Properties on Energetic Contaminant Toxicity

Individual toxicities of TNT and RDX varied across the selected soils. Soil-related differences were evident in acute (adult survival) and chronic (juvenile production) toxicity benchmarks for TNT FA or W-A in each of the five natural soils tested in these studies. Chronic toxicity (the main focus of these studies) to *E. crypticus,* based on the EC_{50} values for TNT FA or W-A in soil, was in the order (from greatest to least toxicity; from smallest to greatest EC_{50} values): TSL > SSL > KCL ≥ RCL > WCL. The effect of soil on TNT toxicity was investigated by determining quantitative relationships between the concentration-response-based toxicity benchmark estimates (EC_{20} and EC_{50}) for acute or chronic endpoints and soil property measurements, shown in Table 1. All linear correlations were performed on the original

(untransformed) data. Pearson's linear correlation coefficients (r) and their respective p-values are summarized in Table 39. There was no statistically significant collinearity ($r = 0.777$; $p = 0.122$) between soil OM and clay content measurements, which are key soil constituents that could affect bioavailability of TNT. Multicollinearity among soil sand, silt, and clay contents ($r \geq -0.950$; $p \leq 0.013$) was present (data are not shown), as expected, due to the methods of determination of these constituents, but it was deemed inconsequential for the purposes of these studies. There was significant correlation between clay content and soil pH ($r = 0.878$; $p = 0.050$; Table 39). However, this covariation was consequential only for the acute EC_{50} estimate for FA TNT (Table 39), which was not the focus of these studies.

Table 39. Pearson Correlation Coefficients for Key Soil Properties and TNT Toxicity Benchmarks for Acute (Adult Survival) and Chronic (Juvenile Production) Endpoints Determined in Definitive Tests with *E. crypticus*

Parameter	Clay Content		OM Content		pH	
	r	*p*	*r*	*p*	*r*	*p*
Clay content	1.000	0.000				
OM content	0.777	0.122	1.000	0.000		
pH	0.878	0.050	0.503	0.388	1.000	0.000
$EC_{20}FA_{accute}$	0.963	0.008	0.898	0.038	0.736	0.157
$EC_{50}FA_{accute}$	0.914	0.030	0.819	0.090	0.892	0.042
$EC_{20}W\text{-}A_{accute}$	0.687	0.200	0.938	0.018	0.291	0.635
$EC_{50}W\text{-}A_{accute}$	0.693	0.195	0.675	0.211	0.294	0.632
$EC_{20}FA_{chronic}$	0.725	0.166	0.992	0.001	0.460	0.436
$EC_{50}FA_{chronic}$	0.721	0.169	0.991	0.001	0.423	0.478
$EC_{20}W\text{-}A_{chronic}$	0.968	0.007	0.671	0.215	0.835	0.078
$EC_{50}W\text{-}A_{chronic}$	0.909	0.033	0.961	0.009	0.648	0.237

Notes: The r values with corresponding probabilities were determined using data from the definitive toxicity tests with SSL, TSL, KCL, RCL, and WCL soils. Estimates of EC producing a 20% (EC_{20}) or 50% (EC_{50}) decrease in the measurement endpoint compared with acetone control were determined for TNT FA and W-A in soil.

Clay content of the soil was strongly ($r \geq 0.909$) and significantly ($p \leq 0.033$) correlated with both (EC_{20} and EC_{50}) acute toxicity benchmarks for TNT FA in soil and with both chronic toxicity benchmarks for TNT W-A in soil (Table 39). Organic matter content of the soil was strongly ($r \geq 0.991$) and significantly ($p = 0.001$) correlated with both chronic toxicity benchmarks for TNT FA in soil, with chronic EC_{50} for TNT W-A in soil ($r = 0.961$; $p = 0.009$), and with acute EC_{20} for FA TNT ($r = 0.898$; $p = 0.038$) or for TNT W-A in soil ($r = 0.938$; $p = 0.018$; Table 39). Significant correlations were also detected for the acute EC_{50} benchmarks for FA TNT and soil pH ($r = 0.892$; $p = 0.042$; Table 39) and CEC values ($r = 0.963$; $p = 0.008$; data not shown in Table 39).

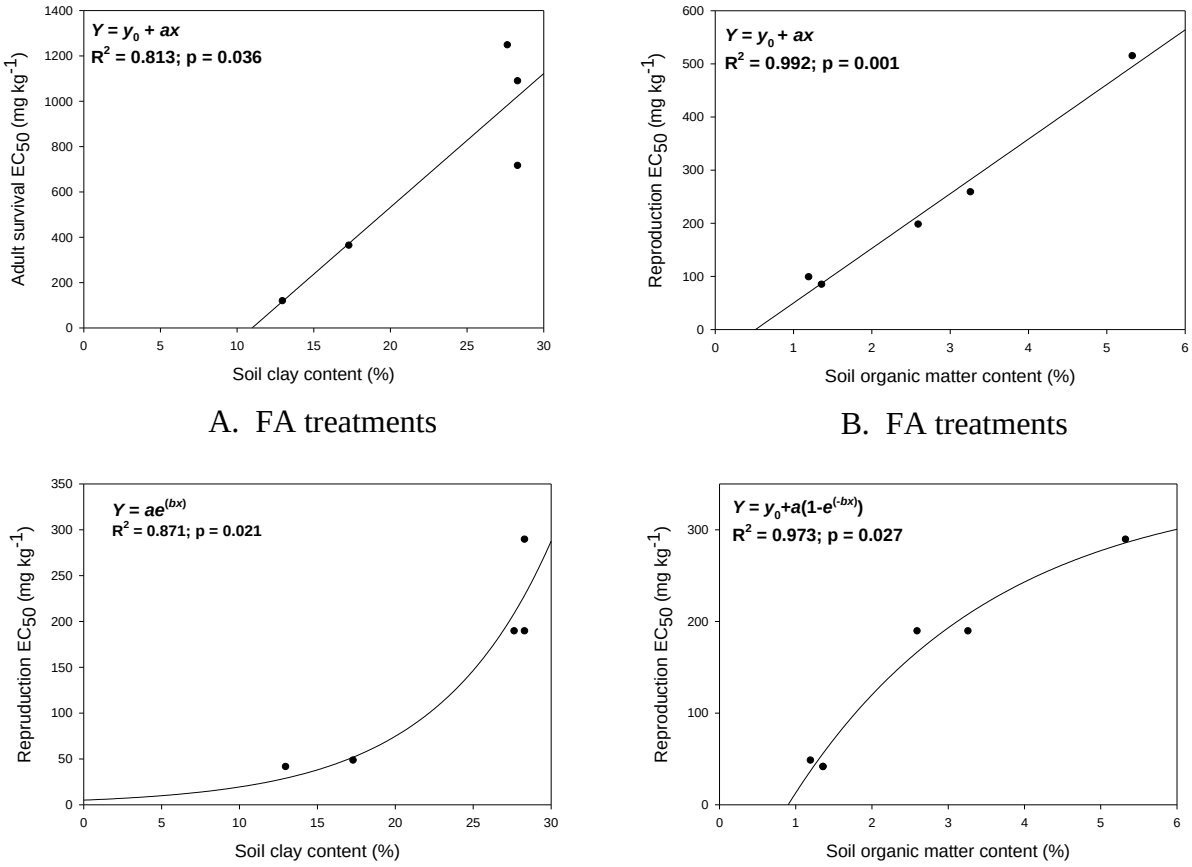

Figure 9. Effects of clay and OM content on toxicity of TNT FA (A and B) or W-A (C and D) in five natural soils to *E. crypticus*.

Results of Pearson's linear correlation analysis were explored further by curve-fitting the highly correlated EC_{50}–soil property pairs with appropriate regression models (Figure 9). The associations between either acute or chronic EC_{50} values with soil clay content (Figure 9A) or OM content (Figure 9B) for FA TNT were best described by linear models. Nonlinear models had the best fit for reproduction toxicity benchmarks for TNT W-A in soils and clay content (Figure 9C; two-parameter single exponential growth model) or OM content (Figure 9D; three-parameter single exponential rise to maximum model). These results revealed both clay and OM contents as the dominant properties modulating the TNT toxicity for *E. crypticus*. The results also suggested that the mechanisms of mitigating effects of soil clay and OM on TNT toxicity change over time, as indicated by a transition from linear to quadratic associations found for TNT FA and W-A in soil, respectively.

Correlation analysis was not performed for RDX effects data because concentration-response relationships for RDX could be quantified only in two out of five experimental soils within the concentration ranges tested. Overall, toxicity of RDX for *E. crypticus* was greater in sandy loam soils compared with clay loam soils.

4. DISCUSSION

Development of ecotoxicological benchmarks for energetic soil contaminants has become a critical need in recent years (Kuperman et al., 2009b). These benchmarks are required for derivation of Eco-SSLs for use in ERA of contaminated sites (USEPA, 2005). The Eco-SSLs represent concentrations of chemicals in soil that, when not exceeded, will be theoretically protective of terrestrial ecosystems within specific soil boundary conditions from unacceptable harmful effects. These values can be used in the SLERA to identify those contaminants that are not of potential ecological concern in soils, and thus do not require further evaluation in the BERA, potentially resulting in cost-savings during ecologically based site assessments and remedial investigations. An extensive review of literature (Kuperman et al., 2009a) provided evidence that there was insufficient information existing for TNT or RDX to generate Eco-SSLs for soil invertebrates. Our toxicity studies, designed to specifically fill these knowledge gaps, used natural soils that meet the criteria for Eco-SSL development, in large part because they have characteristics supporting high relative bioavailability of TNT and RDX. The weathering-and-aging procedure applied to soils amended with a range of TNT or RDX concentrations allowed us to determine the net ecotoxicological effect of complex fate processes in soil that affect bioavailability of TNT and RDX for the soil invertebrate *E. crypticus*, and to more realistically assess the toxicity under conditions more closely resembling the potential exposure effects in the field.

4.1 Analytical Determinations of TNT and RDX in Soil

The exposure concentrations of TNT and RDX in soil were analytically determined at the beginning of each definitive toxicity test using ACN extraction and U.S. EPA Method 8330A (USEPA, 2007). This method quantifies the "total" extractable concentration of each explosive, which includes the nonaccessible (nondissolved crystalline plus adsorbed) and the water-soluble fractions of TNT or RDX. Consequently, the U.S. EPA Method 8330A has the potential to overestimate the amount of explosive available to the exposed organism because the bioavailability of an organic compound having a log $K_{ow} < 5$ (1.6 for TNT and 0.90 for RDX; Monteil-Rivera et al., 2009) for uptake by a soil organism is determined by the fraction dissolved in the soil interstitial water (Belfroid et al., 1994, 1996; Savard et al., 2010). Therefore, in addition to ACN extraction, the water-soluble fraction of TNT was extracted from soil using the ATCLP method (Haley et al., 1993). TNT concentration determined by this method better simulates field soil–water conditions due to respiration by soil biota and was perceived to measure the intensity factor of the bioavailable fraction of chemicals in soil. The ATCLP-based extraction was not applied to RDX because all concentrations selected for toxicity tests with *E. crypticus* exceeded aqueous solubility of RDX (42 mg L^{-1} at 20 °C; Monteil-Rivera et al., 2004), and because the RDX partitioning in the soil interstitial water is expected to control the RDX uptake by soil invertebrates from soil only up to the limit of RDX saturation in the interstitial water (i.e., below 100 mg·kg^{-1}) as was confirmed recently by Savard et al. (2010) for earthworms.

R^2 values for ACN- and ATCLP-based extractions determined in nonlinear regression analyses of the reproduction toxicity data from studies with TNT FA and W-A in soils were compared to determine which chemical measure of exposure correlated better with TNT

toxicity. These comparisons showed that both extraction methods had excellent correlation with the toxicity data for juvenile production, and that neither extraction method provided an advantage for characterizing bioavailability of TNT to *E. crypticus*. This result supports a decision of developing draft Eco-SSLs for TNT for soil invertebrates on the basis of ACN extraction. The ACN extraction-based Eco-SSL values will be especially practical for ERA at contaminated sites because TNT concentrations determined during site characterization are typically based on ACN extraction by U.S. EPA Method 8330A.

Recovery of TNT using ACN extraction was 89 ± 2% (mean ± SE; n = 35) of nominal concentrations across all FA soils tested, indicating good correlation between nominal and measured TNT concentrations determined in our study after a 24 h moisture equilibration period for soils hydrated to 60% of the WHC. A 40% recovery of TNT was determined in the lowest nominal 10 mg kg^{-1} treatment in TSL. These results are consistent with findings by Rocheleau et al. (2006), who reported recoveries above 80% for nominal TNT treatments ranging from 20 to 500 mg kg^{-1} but lower recoveries at nominal TNT treatments of ≤10 mg kg^{-1} in studies with FA SSL soil. Decreased recoveries of TNT in low nominal treatments (below 20 mg kg^{-1}) suggest that a portion of TNT can be rapidly transformed, degraded, or sorbed to soil matrix during the initial 24 h period of soil hydration. These mechanisms are corroborated by the findings of Myers et al. (1998), who reported that sorption of TNT in soils with a wide range of physical properties was rapid and occurred on a time scale of a few minutes. Dodard et al. (2003) reported an average of 99% recovery of TNT from OECD artificial soil amended with a comparable range of nominal TNT concentrations. Such high recovery of TNT can be attributed to both the properties of the components of this formulated soil and the absence or insufficient microbial transformation of TNT in dry OECD artificial soil (treatments were extracted immediately after preparation) compared with hydrated natural soils. Major et al. (1992) suggested that the time-dependent disappearance of explosives may be due to covalent or other nonequilibrium bonding to natural soil components, and therefore, analytical results for soils that were amended with explosives, air-dried, then immediately extracted, test primarily the "potential" efficiency of the extraction process. Overall, our results from chemical analysis confirmed that the soil amendment procedure used in toxicity tests was appropriate, and that U.S. EPA Method 8330A was efficient for quantifying the amount of TNT in soil.

The 3 month weathering-and-aging of TNT in soils decreased TNT concentrations in all soils tested. The residual concentrations were representative of TNT concentrations found in contaminated soils at some former ammunition plants (Simini et al., 1995) and military training ranges (Hewitt et al., 2007; Jenkins et al., 2006, 2007; Walsh et al., 2007). The overall recovery of TNT was 48 ± 5% (mean ± SE; n = 32) of initial concentrations in hydrated FA soils. The recovery of TNT was in the order of (means ± SEs shown): TSL (65 ± 5%) > SSL (61 ± 9%) > WCL (40 ± 13%) > KCL (34 ± 10%) > RCL (32 ± 8%). The resulting TNT concentrations after weathering-and-aging in soils were directly related to soil properties defining the QRB scores for organic chemicals (USEPA, 2005). The amount of TNT remaining in soil after the 3 month weathering-and-aging was the greatest in TSL and SSL, with "very high" QRB scores; intermediate in WCL and KCL, with "medium" QRB scores; and the lowest in RCL, with "low" QRB score, according to the Eco-SSL criteria (Table 1; USEPA, 2005). The rate of decrease was linear or almost linear over the range of concentrations used in sandy loam soils and curvilinear in clay loam soils (Figure 10). Regression analyses showed that both OM content (R^2 = 0.858;

$p = 0.024$) and clay content ($R^2 = 0.939$; $p < 0.0001$) of the soil affected the rates of decrease from the initial TNT concentrations, based on strong associations (Figure 11) of these soil constituents with the slope coefficient values determined for regression curves shown in Figure 10. These results confirmed previously reported findings (Dontsova et al., 2009; Haderlein et al., 1996; Jaenig, 2006; Singh et al., 2008, 2010; Pennington and Brannon, 2002) that identified clay and OM as the key soil constituents that adsorb explosive and thus affect bioavailability and toxicity of TNT for soil organisms. The results of the present studies also show that the mechanisms of mitigating effects of clay and OM on TNT toxicity in tested soils change over time, as indicated by a transition from linear to quadratic associations found for TNT FA and W-A in soil, respectively.

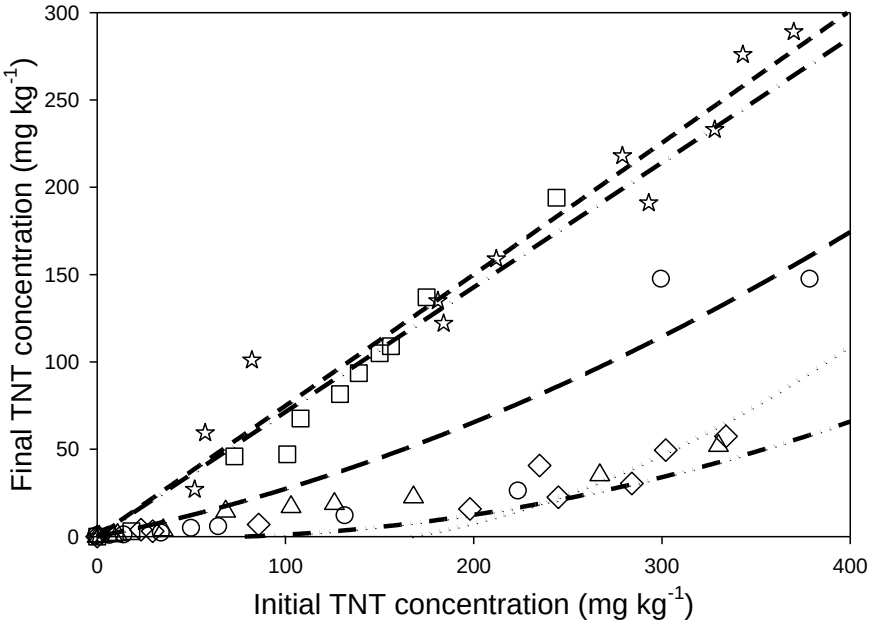

Figure 10. Relationships between initial TNT concentrations in FA soils and final TNT concentrations after the 3 month weathering-and-aging in soils. Initial concentration versus that due to W-A in TSL (☆ data points, - - -), SSL (☐ data points, ─ • ─), KCL (○ data points, ─ ─ ─), RCL (△ data points, ─ •• ─), and WCL (◇ data points, •••) determined after a 24 h moisture equilibration of FA soil hydrated to 60% of the WHC of each soil.

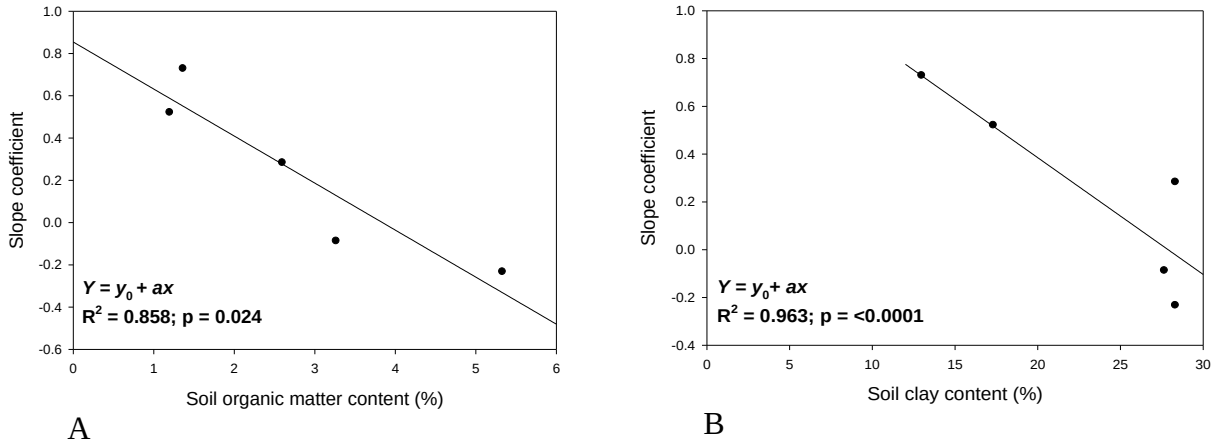

Figure 11. Relationships between the slope coefficient values and OM (A) or clay (B) content of natural soils. Values for slope coefficients were determined in regression analyses shown in Figure 10.

Within the soils, percent recovery was directly related to the initial TNT concentration in amended treatments. Comparable results were found in studies with TNT and related nitroaromatic compounds (NACs) including 1,3,5-trinitrobenzene (TNB); 2,4-dinitrotoluene (2,4-DNT); and 2,6-dinitrotoluene (2,6-DNT) W-A in natural soils under similar conditions (Kuperman et al., 2004, 2005, 2006b; Renoux et al., 2000; Rocheleau et al., 2006, 2010). Abiotic factors that can affect the fate of TNT in soil including photolysis, moistening and air-drying cycles, and temperature were controlled in our studies and were similar in all treatments. Therefore, possible explanations of the observed relationship between percent recovery of TNT and the initial concentration in FA soil could be the inhibition of microbial activity due to toxicity at greater TNT concentrations and increasing saturation of binding sites of the soil constituents (Rocheleau et al., 2006; Dodard et al., 1999). Overall, chemical analyses demonstrated that TNT exposure conditions of *E. crypticus* in amended soils subjected to weathering-and-aging procedures differed from those of FA soils. The inclusion of these procedures in the assessments of TNT toxicity to *E. crypticus* allowed us to incorporate potential alterations in chemical bioavailability and resulting toxicity at contaminated sites into the development of toxicological benchmarks for soil invertebrates.

In contrast with the fate of TNT in amended soils, RDX concentrations in the five soils tested in our studies did not appreciably decrease during the 3-month weathering-and-aging process. These results are consistent with other studies that investigated fate and ecotoxicological effects of RDX in soils under aerobic conditions. RDX recoveries averaging 93, 95, and 83% after similarly performed weathering-and-aging procedures using SSL soil were determined by Kuperman et al. (2003), Rocheleau et al. (2005), and Simini et al. (2006), respectively. Sheremata et al. (2001) reported little RDX degradation under aerobic conditions in batch cultures in a natural soil. Extensive degradation occurred only under anaerobic conditions after several weeks; RDX metabolites hexahydro-3,5-dinitro-1-nitroso-1,3,5-triazine (MNX), hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine (DNX), and hexahydro-1,3,5-trinitroso-1,3,5-

triazine (TNX), were not found until after extensive anaerobic degradation had occurred. The authors also measured relatively low sorption (K_d^s) values (0.83 L kg^{-1}), although the sorption that occurred was nearly irreversible (Sheremata et al., 2001; Checkai et al., 1993). Sorption of RDX to soils, as demonstrated by low K_d values, is low, and RDX is therefore highly mobile in soils, governed by interactions with soil minerals rather than by association with soil organic matter (Monteil-Rivera et al., 2009). Consequently, RDX is readily leached through the vadose zone, which presents a high risk for groundwater contamination.

4.2 Toxicities of TNT and RDX in Natural Soils

This project was undertaken to produce scientifically defensible toxicity data for the development of soil invertebrate-based Eco-SSL values for TNT and RDX, and to investigate and characterize predominant soil physicochemical parameters that can affect the bioavailability and resulting toxicity of TNT or RDX to soil invertebrates. To achieve the first objective, studies were designed to meet specific criteria (USEPA, 2005). Eco-SSL test acceptance criteria were met or exceeded in these investigations by ensuring that:

(1) Tests were conducted in natural soils having physicochemical characteristics that support high relative bioavailability of TNT or RDX;
(2) Experimental designs for laboratory studies were documented and appropriate;
(3) Both nominal and analytically determined concentrations of chemicals of interest were reported;
(4) Tests included both negative and positive controls;
(5) Chronic or life cycle tests were used;
(6) Appropriate chemical dosing procedures were reported;
(7) Concentration-response relationships were reported;
(8) Statistical tests used to calculate the benchmark and level of significance were described; and
(9) The origin of test species was specified and appropriate.

Definitive studies using the potworm *E. crypticus* exposures in TSL soil established new ecotoxicological data for TNT effects on soil invertebrates under conditions of very high relative bioavailability for organic chemicals in soil (as defined in USEPA, 2005), and confirmed the toxicity benchmarks established for TNT in our previous studies with SSL soil (Kuperman et al., 2004a, 2005, 2006e). Acute toxicities of TNT (either FA or W-A in soil, respectively) were not statistically different between TSL and SSL, based on the EC_{20} values and respective 95% CIs for adult survival, although they varied at the EC_{50} level (Tables 19 and 21). Reproduction toxicity of TNT either FA or W-A in soil was similar for TSL and SSL, based on the EC_{20} or EC_{50} values (Tables 20 and 22). The similarity of ecotoxicological effects of TNT in the two soils was also evident from the presence of hormetic stimulation of juvenile production by *E. crypticus* at low TNT concentrations (<100 mg kg^{-1}) in FA soils (Figures 1 and 2) and from the toxicity-enhancing effect of weathering-and-aging of TNT in soil, which doubled the reproduction toxicities for the potworms in TSL and SSL soils (Tables 20 and 22).

Results of the present studies with TSL confirmed ecotoxicological data established for RDX in previous studies with SSL soil (Kuperman et al., 2003, 2004), and showed that RDX was less toxic to *E. crypticus* compared with TNT toxicity in the same soil types. This contrasted with findings of Simini et al. (2003, 2006) who reported greater toxicity of RDX to the earthworm *Eisenia fetida* in FA SSL soil compared with toxicity of TNT to *E. crypticus* in a similar SSL soil. However, Simini's order of RDX and TNT toxicities were reversed after EM weathering-and-aging in SSL and became consistent with the results of present studies with *E. crypticus*. Acute RDX toxicity was greater in the present study with FA TSL compared with FA SSL (Tables 34 and 35), but the difference disappeared after weathering-and-aging of RDX in the respective soils, producing the unbounded NOEC values for adult survival equal to the greatest RDX concentrations tested (Tables 34 and 35). Reproduction toxicity of RDX (either FA or W-A in each soil, respectively) was statistically similar for TSL and SSL, based on the EC_{20} or EC_{50} values and respective 95% CIs (Tables 34 and 35).

Toxicological benchmarks for TNT established in the present studies with *E. crypticus* were generally consistent with data for soil invertebrates reported in a comprehensive review by Kuperman et al. (2009a). The chronic EC_{50} values for TNT summarized in Kuperman et al. (2009a) ranged from 23 to 919 mg kg^{-1} for different soil types, test species, and degree of weathering-and-aging of TNT in soil, and were derived using either nominal or analytically determined soil TNT concentrations. Dodard et al. (2003) determined the EC_{20} and EC_{50} values for TNT of 59 and 111 mg kg^{-1} for juvenile production by *E. albidus* in OECD artificial soil, both of which were similar to those determined in our study with FA TSL or SSL soil. In a study with multiple soil types, Schäfer (2002) found that *E. crypticus* was less sensitive to TNT exposure compared with *F. candida* and determined the respective reproduction EC_{50} values of 501 and 64 mg kg^{-1} in Lufa 2.2 soil (2.2% organic C), and 277 and 23 mg kg^{-1} in Lufa 2.3 soil (0.7% organic C). However, these values may not be representative, due to problems encountered with the performance of both test organisms in soils used in those studies, including failure to meet validity criteria in several control treatments, high data variability, and low reproduction rates of the species tested (Schäfer, 2002). Exposure to TNT in OECD artificial soil affected survival of the earthworm *Eisenia andrei* adults, with the 14 day LC_{50} value of 365 mg kg^{-1} (Robidoux et al., 1999), and reproduction, with the 56 day LOEC value of 111 mg kg^{-1} (Robidoux et al., 2000). A greater toxicity of TNT (compared to values in OECD artificial soil), with the EC_{20} value for juvenile production of 52 mg kg^{-1}, was determined by Robidoux et al. (2002) for *E. andrei* exposed in a sandy forest soil (3.8% OM, 83% sand, 8% clay, 7.6 pH). These results were comparable with findings for *E. crypticus* in the present studies with FA TSL or SSL soils. Acute and subacute (weight change) effects of TNT on the earthworm *E. fetida* have been reported by Phillips et al. (1993) for exposures in SAS and in a natural forest soil that had 5.9% OM content. In those studies, the LOEC values based on the earthworm weight loss and nominal TNT concentrations were 140 and 150 mg kg^{-1} for SAS and forest soil, respectively. Phillips et al. (1993) also reported 100% mortality of *E. fetida* in SAS fortified with a mixture of EMs that included 30, 50, 62.5, and 20 mg kg^{-1} of TNT, TNB, 2,4-DNT, and 2,6-DNT, respectively. Statistically significant ($p < 0.01$) subacute effects on the earthworm (weight loss) were observed at concentrations 6, 10, 12.5, and 4 mg kg^{-1} of TNT, TNB, 2,4-DNT, and 2,6-DNT, respectively. These results showed a greater toxicity of TNT compared with the present studies; however, direct comparisons of data from among these studies are inappropriate due to differences in the experimental designs and particularly due to presence of EM contaminant mixtures in Phillips et al. (1993).

The juvenile production endpoint used in the present studies was a more sensitive measure of EM toxicity to *E. crypticus* in all soils tested compared with the adult survival endpoint. This comports with results reported in literature for potworms (Dodard et al., 2003; Schäfer, 2002; Schäfer and Achazi, 1999; Kuperman et al., 1999, 2003, 2004a, 2004b, 2005, 2006b, 2006c, 2006e), earthworms (Phillips et al., 1993; Robidoux et al., 2000, 2001, 2002; Simini et al., 2003, 2006), and Collembola (Schäfer, 2002; Schäfer and Achazi, 1999). This finding supported the Eco-SSL requirement of using reproduction endpoints for toxicity benchmark development (USEPA, 2005). Overall, the present definitive studies using *E. crypticus* exposures in TSL or SSL soils developed ecotoxicological benchmarks for TNT and RDX in compliance with Eco-SSL test acceptance criteria (USEPA, 2005), and thus achieved the first objective of this investigation. These benchmarks will contribute to the dataset that will be used to derive the soil invertebrate-based draft Eco-SSL values for TNT and RDX. Consequently, these draft Eco-SSL values can be compared to corresponding soil screening concentrations (SSCs) derived from ecotoxicological benchmarks determined for the RCL, KCL, and WCL soils to test the hypothesis that the magnitude of SSC, including Eco-SSL, for each explosive, can be generally predicted from the respective site-specific soil properties, and to assess the level of consistency and relative importance for TNT and RDX of the Eco-SSL classification of soils within specific boundary conditions regarding the bioavailability of organic compounds in upland aerobic soils (USEPA 2005).

4.3 Effects of Soil Properties on TNT or RDX Toxicities

The important role of soil properties in affecting bioavailability and toxicity of energetic soil contaminants to soil invertebrates has been emphasized in several studies (Kuperman et al., 2003, 2004a, 2005, 2006b, 2006c, 2006e; Schäfer, 2002; Simini et al., 2003, 2006; Phillips et al., 1993; Robidoux et al., 2002). To achieve the second objective of the present studies, toxicity testing was conducted with additional natural soils, including KCL, RCL, and WCL, to extend the range of soil physicochemical characteristics that were hypothesized to affect the EM toxicity to soil invertebrates. The QRB scores for organic chemicals in natural soils were considered "very high" for TSL and SSL, "medium" for KCL and WCL, and "low" for RCL soil, according to the Eco-SSL criteria (USEPA, 2005). Soil-related differences were evident in acute (adult survival) and chronic (juvenile production) toxicity benchmarks for TNT FA or W-A in each of the five natural soils tested in our studies. Acute toxicity of TNT for *E. crypticus* was generally greater in the light-textured sandy loam soils compared with the heavier-textured clay loam soils, but the exact order of acute toxicity varied between FA and W-A treatments of TNT in soil. The order of acute toxicity based on the EC_{50} values for TNT FA in soil (from greatest to least toxicity; from smallest to largest EC_{50} values) was TSL > SSL > KCL > WCL > RCL and closely paralleled the QRB scores. The order of acute toxicity for TNT W-A in soil was SSL > TSL > RCL > WCL > KCL. The order of chronic toxicity to *E. crypticus* based on the EC_{50} values for TNT FA or W-A in soil was TSL > SSL > KCL ≥ RCL > WCL, which once again closely paralleled the QRB scores.

The quantitative analyses of relationships among the acute or chronic toxicity benchmarks for TNT and soil property measurements revealed that both the clay and OM contents of the soil affected the toxicity of TNT to *E. crypticus*, with few distinctions based on specific exposure conditions and toxicity endpoints. Clay content correlated strongly with acute

toxicity benchmarks for TNT FA in soil and with chronic toxicity benchmarks for TNT W-A in soil (Table 39). Soil OM content correlated strongly with chronic toxicity benchmarks for TNT FA in soil, with chronic EC_{50} values for TNT W-A in soil, and with acute EC_{20} values for TNT FA or W-A in soil (Table 39). Significant correlations were also detected for the acute EC_{50} benchmarks for FA TNT and soil pH or CEC values. These results identified OM content as the dominant property mitigating TNT toxicity for juvenile production by *E. crypticus* in FA soil. Both clay and OM contents of the soil modulated reproduction toxicity of TNT W-A in soil, which confirmed the importance of including weathering-and-aging of EMs in soil into experimental designs of toxicity tests that aim to assess the effects of soil properties on bioavailability and toxicity of EMs in diverse field soils at historically contaminated sites.

Results of the present studies comport with the findings of several published studies that suggest bioavailability of TNT and related NACs can be affected by clay content (Emery et al., 2001; Haderlein et al., 1996; Singh et al., 2008), OM content (Anzhi et al., 1997; Eriksson and Skyllberg, 2001; Singh et al., 2010), or a combination of the two (Jaenig, 2006). Sorption of NACs, and particularly of TNT, to constituents of natural soils is not linear, and is dominated by strong and specific interactions with certain matrix components, rather than by hydrophobic partitioning (Monteil-Rivera et al., 2009). Among all matrix components commonly found in soils including clays, carbonates, quartz, aluminum, iron (hydr)oxides, and OM, clays were found to be strong sorbents for NACs (Daun et al., 1998; Esteve-Núñez et al., 2001; Haderlein et al., 1996; Weissmahr et al., 1997, 1998). TNT and other NACs can be sorbed to uncharged regions of phyllosilicate clays through electron donor-acceptor complexes between oxygen atoms of the siloxane surface and the six-carbon ring of TNT, through pi-bonding (Haderlein et al., 1996; Weissmahr et al., 1998). Consequently, the adsorption of NACs can be strongly affected by exchangeable cations (Haderlein et al., 1996), which may partially explain significant correlation between the acute EC_{50} benchmark for FA TNT and soil CEC found in the present studies. In aqueous environments, adsorption of the NACs to clays is high when the exchangeable cations at the clays are a mixture that includes K^+ and NH_4^+, but is negligible for homoionic Na^+–, Ca^{2+}–, Mg^{2+}–, and Al^{3+}– clays (Haderlein et al. 1996; Weissmahr et al., 1997). Furthermore, the affinity and the adsorption capacity of the clays for NACs increase in the order kaolinite < illite < montmorillonite. Thus, clay minerals, plus their abundance and degree of K^+– and NH_4^+– saturation, can control the phase distribution and bioavailability of NACs in soils (Haderlein et al., 1996).

TNT and its metabolites were also shown to react and sorb to OM in the soil (Achtnich et al., 1999; Anzhi et al., 1997; Dawel et al., 1997; Drzyzga et al., 1998; Eriksson and Skyllberg, 2001; Esteve-Núñez et al., 2001; Simpson, 2006; Singh et al., 2010; Thorn and Kennedy, 2002; Thorn et al., 2002; Xing and Pignatello, 1997; Weiß et al., 2004). Sorption studies with low-polarity organic compounds, including nitroaromatic energetic materials, have shown that binding of these compounds to both soil OM (Xing and Pignatello, 1997) and silicate clays (Haderlein et al., 1996) is competitive, selective, nonlinear, and frequently reversible. Both specific (electrostatic interactions/covalent bond formation reactions between functional groups in the organic contaminant and OM) and nonspecific (hydrophobic partitioning reaction between nonpolar organic contaminants and nonpolar moieties of OM) adsorption mechanisms are possible in TNT and soil OM. In a study by Singh et al. (2010), the carbonyl carbon content of OM was responsible for 98% variation in TNT sorption, possibly because positively charged

carbonyl carbon is electrostatically associated with the negatively charged nitro groups of TNT. The importance of negatively charged functional groups in TNT adsorption was confirmed by an increase in TNT sorption to humic acids with an increase in soil pH and, therefore, with dissociation of hydroxylic and carboxylic groups in organic carbon (OC) (Li et al., 1997; Ainsworth et al., 1993; Eriksson and Skyllberg, 2001). This finding is consistent with significant correlation between the acute EC_{50} benchmark for FA TNT and soil pH determined in the present studies.

Toxicity of RDX for *E. crypticus* was greater in the light-textured sandy loam soils (TSL and SSL) compared with the heavier-textured clay loam soils (RCL, WCL, and KCL). These results were generally consistent with findings of Savard et al. (2010), who reported that variations in RDX bioavailability for the earthworm *E. andrei*, measured as the biota soil accumulation factor (BSAF) values, were soil-specific and decreased in the order of Defence Research and Development Canada (Canadian sandy soil; 1% clay, 1.2% OM) > TSL (similar to soil used in our studies) > KCL (19% clay, 1.5% OM) > WCL (similar to soil used in our studies) at RDX concentrations ≤100 mg kg^{-1}. The smallest BSAF was determined for WCL soil, which had the greatest OM content and the lowest RDX bioavailability among soils tested in those studies. However, at RDX concentrations in soil ranging from 100 to 10,000 mg kg^{-1}, which were more relevant to the results of our studies, the trend was less clear (Savard et al., 2010).

Several studies have shown that although RDX had low tendency for sorption, its sorption was governed by the clay content of the soil (Balakrishnan et al., 2004; Leggett, 1985; Monteil-Rivera et al., 2009; Sheremata et al., 2001). Contrasting with this conclusion was the significant linear regression between K_d values and soil OC content determined by Tucker et al. (2002) and findings by Dontsova et al. (2009), which indicated that adsorption to OC is the main mechanism of RDX interaction with the soils. Notwithstanding the difference in proposed mechanisms contributing to the fate of RDX in soil, it is likely that RDX was less bioavailable in KCL, RCL, and WCL soils because of the greater clay and OM contents of these soils, compared with the sandy loam soils (TSL and SSL) used in the present studies. The quantitative relationships among the toxicity benchmarks for RDX and soil property measurements could not be determined because concentration-response relationships for RDX were established only in two out of five experimental soils used in the present studies, due to the low toxicity of RDX to potworms.

4.4 Effects of Weathering-and-Aging Explosives in Soil on Toxicity

In addition to contaminant loading, the attainment of sorption-desorption equilibria for EM and their transformation in contaminated soils are time-dependent processes that ultimately determine EM bioavailability to soil organisms. These processes can decrease the amount of chemical that is bioavailable as compared with freshly contaminated soils or may increase toxicity due to the presence of more toxic transformation products than parent compound freshly introduced into soil (Alexander, 2000; Hawari et al., 1998, 2000; Preuß and Rieger, 1995; Gorontzy, et al., 1994; Spain, 2000; Schäfer, 2002; Kaplan, 1992; Kuperman et al., 2005; 2006b; 2006e; Sunahara et al., 2001). Therefore, the present studies included weathering-and-aging of TNT or RDX in soil in the experimental designs to determine the net

ecotoxicological effects of these complex processes and to more closely approximate the exposure effects in the field. These studies revealed alterations in toxicity for *E. crypticus* after weathering-and-aging of TNT or RDX in soil, and these alterations were soil- and endpoint-specific. Weathering-and-aging of TNT in soil significantly decreased acute toxicity for *E. crypticus* in TSL but significantly increased acute toxicity in SSL and RCL soils, compared with respective toxicities in FA soils, based on the EC_{50} values for adult survival and the respective 95% CIs (Tables 19, 21, and 25). Weathering-and-aging of TNT in soil significantly increased chronic toxicity for *E. crypticus* in all soils except KCL, compared with respective toxicities in FA soils, based on either the EC_{20} or EC_{50} values for juvenile production and the respective 95% CIs (Tables 20, 22, 24, 26, and 28). Weathering-and-aging of RDX in soil significantly (95% CI basis) decreased both acute and chronic toxicities for *E. crypticus* in TSL (Table 34), but did not affect RDX toxicity in other soils tested in the present studies.

As discussed previously, different products that are formed during biotic and abiotic transformation of TNT in soil under aerobic conditions can alter the exposure effects for *E. crypticus* compared with those effects that can be observed when parent compound is freshly introduced into soil. These transformation products can include 2-amino-4,6-dinitrotoluene (2-ADNT), 4-amino-2,6-dinitrotoluene (4-ADNT), 2,4-diaminotoluene (2,4-DANT), and 2,6-diaminotoluene (2,6-DANT) (Ainsworth et al., 1993; Dodard et al., 2004; Esteve-Núñez et al., 2001; Fernando et al., 1990; Hawari et al., 2000; McCormick et al., 1976; Monteil-Rivera et al., 2009). In addition, 2,4-DNT and 2,6-DNT are common byproducts found in munitions as impurities resulting from TNT manufacturing (Major et al., 2002). The reduction of amines proceeds through the formation of nitroso derivatives (ArNO; 2-NO-DNT and 4-NO-DNT), and hydroxylamine derivatives (ArNHOH; 2-HADNT and 4-HADNT), which can be transformed further to azoxy-TNT compounds (Monteil-Rivera et al., 2009; and references therein). Photolysis of TNT can lead to formation of additional NACs including 3,5-dinitroaniline (3,5-DNA); 2,4,6-trinitrophenol (TNP); 2,4,6-trinitrobenzyl alcohol; 2,4,6-trinitrobenzoic acid; and TNB (Monteil-Rivera et al., 2009). Several of these products were found in TNT-contaminated soil (Daun et al., 1998; Frische, 2002), and a few including 2-ADNT; 4-ADNT; 2,4-DANT; and 2,6-DANT have been detected in earthworms *E. andrei* and *Lumbricus terrestris* exposed to TNT-contaminated soils (Johnson et al., 2000; Renoux et al., 2000; Robidoux et al., 2000). Some of these NACs are potentially more bioavailable and/or toxic than their precursors (Rieger and Knackmuss, 1995). Kuperman et al. (2006b) reported greater toxicities of TNB; 2,6-DNT; and 2,4-DNT compared with TNT toxicity to *E. crypticus* in SSL soil. In a study investigating relative toxicities of TNT and its reduced metabolites in a sandy loam forest soil (8% clay, 3.8% OM), Lachance et al. (2004) demonstrated that acute toxicity of 4-ADNT to the earthworm *E. andrei* was greater compared with the toxicity of parent compound, whereas exposures to equimolar concentrations of 2-ADNT; 2,4-DANT; and 2,6-DANT were less toxic compared with TNT for *E. andrei* adults. Presence of 2-ADNT and 4-ADNT was detected in our previous similarly designed studies at all concentrations of TNT W-A in SSL soil, but in greater amounts at concentrations between 50 and 200 $mg \cdot kg^{1}$ (Rocheleau et al., 2006). The aminonitrotoluene intermediates can be formed by soil bacteria in either aerobic or anaerobic conditions (Hawari et al., 1998, 2000; Monteil-Rivera et al., 2009). They are the most commonly detected products of TNT transformation and can contribute to alteration of toxicity for *E. crypticus* after weathering-and-aging of TNT in natural soils. Identification of products of TNT transformation in soils subjected to the weathering-and-aging procedure was not included

in the scope of the current investigation because we focused primarily on the net toxic effects of exposure of *E. crypticus* to TNT in aerobic upland soils. Our ongoing studies with aminonitrotoluene intermediates of TNT transformation will provide additional information required to resolve definitively the current uncertainties in our knowledge of relative toxicities of TNT and its transformation products, especially as they relate to chronic exposure effects for soil invertebrates.

The net effects of weathering-and-aging of contaminant EM in soil on the resulting exposure effects for soil invertebrates were investigated in several studies (Kuperman et al., 2003, 2004a, 2005, 2006b, 2006c, 2006e; Schäfer, 2002; Simini et al., 2003, 2006). Kuperman et al. (2005, 2006b, 2006c) reported that weathering-and-aging in SSL soil significantly increased the toxicities of TNT; 2,6-DNT; and 2,4,6,8,10,12-hexanitro-2,4,6,8,10,12-hexaazaisowurtzitane (CL-20) to *E. crypticus*, whereas the toxicities of 2,4-DNT or TNB were unaffected. In contrast, a decreased toxicity of TNT after aging in soil was reported for *E. albidus* in OECD artificial soil (Dodard et al., 2003) and for *Folsomia candida* in Lufa 2.2 soil (Schäfer, 2002) or SSL soil (Kuperman et al., 2006f). No effects of weathering-and-aging of RDX or octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) in SSL soil on toxicity were reported for *E. crypticus* (Kuperman et al., 2003) and *E. fetida* (Simini et al., 2003). Several factors may contribute to differential effects of TNT weathering-and-aging in soils on the toxicity to *E. crypticus* observed in the present studies, and the toxicity to *E. crypticus* or *F. candida* determined in studies by Dodard et al. (2003) and Schäfer (2002). These include differences in the properties of soils used in the studies, the weathering-and-aging procedures employed (including soil aerobicity during processing and testing), and resulting effects on the bioavailability of TNT and its transformation products for the organisms tested. Additional studies would be required to resolve the current uncertainties in our understanding of the mechanisms contributing to the increased or decreased toxicity of EMs following their weathering-and-aging in soil. These studies should be conducted with different soil types having ranges of properties that affect the fate and bioavailability of EMs in order to better understand the complex interactions among physical, chemical, and biological components that jointly contribute to the outcome of ecotoxicity testing.

5. CONCLUSIONS

This project was undertaken to produce scientifically defensible toxicity data for the development of soil invertebrate-based Eco-SSL values for TNT and RDX, and to investigate and characterize predominant soil physicochemical parameters that can affect the bioavailability and resulting toxicities of TNT or RDX to soil invertebrates. The present studies produced ecotoxicological data for TNT and RDX using the ecologically relevant soil invertebrate species *E. crypticus*. Reproduction was a more sensitive endpoint for evaluation of the exposure effects compared with adult survival; therefore, reproduction endpoint-based toxicity benchmarks should be used to set screening criteria for TNT and RDX. This finding also supports the Eco-SSL requirement of using reproduction endpoints for toxicity benchmark development (USEPA, 2005).

The natural soils TSL and SSL were used in toxicity tests to develop ecotoxicological data for use in derivation of Eco-SSLs. These soils had low OM and clay contents, which fulfilled the U.S. EPA requirement of using soil with characteristics that support high relative bioavailability of organic contaminants for developing realistic yet conservative Eco-SSL values (USEPA, 2005). The exposure concentrations of TNT or RDX in soil were analytically determined at the beginning of each definitive toxicity test; consequently, the ecotoxicological benchmarks were determined using measured TNT or RDX concentrations. This complied with the U.S. EPA preference for derivation of Eco-SSL values on the basis of measured soil concentration of a chemical over those based on nominal concentrations (USEPA, 2005). Chemical analyses of FA soils using U.S. EPA Method 8330A showed generally good correlation between nominal and measured ACN-extracted concentrations, which confirmed that the soil amendment procedure used in the definitive toxicity tests was appropriate, and that this method was efficient for quantifying the amounts of TNT or RDX in soil. Overall, the definitive studies using *E. crypticus* exposures in TSL or SSL soils supported the development of ecotoxicological benchmarks for TNT and RDX in compliance with Eco-SSL test acceptance criteria (USEPA, 2005); thus, the first objective of this investigation was achieved. All ecotoxicological benchmarks determined in these studies will be provided to the Eco-SSL Work Group for quality-control review before inclusion in the Eco-SSL database and for subsequent use in the development of individual soil invertebrate-based Eco-SSL values for TNT and RDX.

In addition to ACN extraction, the water-soluble fraction of TNT was extracted from soil using the ATCLP method, which was perceived to measure the intensity factor, which is the immediately bioavailable fraction of chemical in soil pore water. The present studies showed that extraction by both methods resulted in excellent correlation with the toxicity data for juvenile production, and that neither extraction method had an advantage for characterizing bioavailability of TNT to *E. crypticus*. These results support a decision to develop draft Eco-SSL values for TNT for soil invertebrates on the basis of ACN extraction. The ACN extraction-based Eco-SSL values will be especially practical for ERA at contaminated sites because TNT concentrations determined during site characterization are typically based on ACN extraction and U.S. EPA Method 8330A.

Toxicity testing was conducted using natural soils with a range of physicochemical characteristics that were hypothesized to affect the EM toxicity to soil invertebrates. Soil-related differences were evident in acute and chronic toxicity benchmarks for TNT. The order of chronic toxicity (the main focus of these studies) to *E. crypticus* based on the EC_{50} values for TNT FA or W-A in soil was (from greatest to least toxicity): TSL > SSL > KCL ≥ RCL > WCL. Toxicity of RDX for *E. crypticus* was also greater in sandy loam soils (TSL and SSL) compared with clay loam soils (RCL, WCL, and KCL). But quantitative relationships between the toxicity and soil property measurements could not be determined for RDX because concentration-response relationships for RDX were established only in two out of five tested experimental soils due to its low toxicity to potworms. Analyses of quantitative relationships between the toxicity benchmarks for TNT and soil property measurements identified OM as the dominant property mitigating TNT toxicity for juvenile production by *E. crypticus* in FA soil. These analyses also revealed that both the clay and OM contents of the soil can modulate the toxicity of TNT W-A in soil, which confirmed the importance of inclusion of weathering-and-aging of EMs in soil into experimental designs of toxicity tests that aim to assess the effects of

soil properties on bioavailability and toxicity of EMs in diverse field soils at historically contaminated sites.

The present studies included weathering-and-aging of TNT or RDX in soil in the experimental designs to produce a soil microenvironment more similar to field conditions, and thus more closely approximate the exposure effects in contaminated sites. Results of chemical analyses showed that exposure conditions of *E. crypticus* to EM W-A in soils differed from those of FA soil. Toxicity alterations after the weathering-and-aging process were soil- and endpoint-specific. Weathering-and-aging of TNT in soil decreased acute toxicity for *E. crypticus* in TSL but increased acute toxicity in SSL or RCL soils compared with respective toxicities in FA soils. Weathering-and-aging of TNT in soil increased chronic toxicity for *E. crypticus* in all soils except KCL compared with respective toxicities in FA soils. Weathering-and-aging of RDX in soil decreased both acute and chronic toxicities for *E. crypticus* in TSL but did not affect RDX toxicity in other soils tested in the present studies. Overall, the results of the present studies showed that special consideration given to the effects of weathering-and-aging of EM in soil for assessing toxicity was well justified. Toxicity benchmarks generated in the present studies will contribute to development of Eco-SSL values that better represent the exposure conditions of soil invertebrates at contaminated sites. Our findings of increased reproduction toxicity for *E. crypticus* of TNT W-A in soil and findings reported in the literature clearly show that additional studies are required to more completely investigate and resolve the toxicity of the TNT transformation and degradation products. Analogously, further investigation of the more-toxic transformation compounds that arise within soils amended with TNT should also have a weathering-and-aging component so that the level of persistence and long-term impact of the ecotoxicity of these toxic transformation products may also be assessed. Such studies should also be designed to generate benchmark data for EM transformation products so that results may be used in deriving draft Eco-SSL values for these chemicals while providing more complete information on the ecotoxicological effects of energetic contaminants in soil for risk assessors and site managers.

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ABBREVIATIONS AND ACRONYMS

ACN	acetonitrile
2-ADNT	2-amino-4,6-dinitrotoluene
4-ADNT	4-amino-2,6-dinitrotoluene
AS	artificial soil
ATCLP	adapted toxicity characteristic leaching procedure
BDL	below detection limit
BERA	baseline ecological risk assessment
BSAF	biota soil accumulation factor
CAS	Chemical Abstracts Service
CEC	cation exchange capacity
CI	confidence interval
CL-20	2,4,6,8,10,12-hexanitro-2,4,6,8,10,12-hexaazaisowurtzitane
CV	coefficient of variation
2,4-DANT	2,4-diaminotoluene
2,6-DANT	2,6-diaminotoluene
3,5-DNA	3,5-dinitroaniline
2,4-DNT	2,4-dinitrotoluene
2,6-DNT	2,6-dinitrotoluene
DNX	hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine
DOD	U.S. Department of Defense
EC_{20}	concentration producing a 20% decrease in measurement endpoint
EC_{50}	concentration producing a 50% decrease in measurement endpoint
Eco-SSL	ecological soil screening level
ECp	estimate of effect concentration for a specified percent effect
EM	energetic material
EPA	Environmental Protection Agency
ERA	ecological risk assessment
FA	freshly amended
FLSD	Fisher's least-significant difference
HMX	octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (high melting explosive)
HPLC	high-performance liquid chromatography
ISO	International Organization for Standardization
KCL	Kirkland clay loam
K_{d}^{s}	soil adsorption coefficient
LOAEC	lowest-observed-adverse-effect concentration
LOEC	lowest-observed-effect-concentration
MNX	hexahydro-3,5-dinitro-1-nitroso-1,3,5-triazine
NA	not applicable
NAC	nitroaromatic compound
NOAEC	no-observed-adverse-effect concentration
NOEC	no-observed-effect concentration
OC	organic carbon
OECD	Organisation for Economic Co-operation and Development

OM	organic matter
p	probability value
PAR	photosynthetically active radiation
PTFE	polytetrafluoroethylene
QRB	qualitative relative bioavailability
r	correlation coefficient
R^2	coefficient of determination
RCL	Richfield clay loam
RDX	hexahydro-1,3,5-trinitro-1,3,5-triazine (royal demolition explosive)
SAS	standard artificial soil
SE	standard error
SLERA	screening level ecological risk assessment
SSC	soil screening concentration
SSL	Sassafras sandy loam
TCLP	toxicity characteristic leaching procedure
TNB	1,3,5-trinitrobenzene
TNP	2,4,6-trinitrophenol
TNT	2,4,6-trinitrotoluene
TNX	hexahydro-1,3,5-trinitroso-1,3,5-triazine
TSL	Teller sandy loam
W-A	weathered-and-aged
WCL	Webster clay loam
WHC	water-holding capacity


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